The price of defence: maternal effects in an aposematic ladybird

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: …

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Abstract

Offspring phenotype can be adaptively altered via maternal non-genetic inheritance. Such ‘maternal effects’ enable females to adjust their per offspring investment in response to variation in the offspring environment, and thus maximise their reproductive success. Consequently they play a pivotal role in population dynamics and the response of species to environmental change. Despite this, little is known about how maternal effects mediate reproductive investment in response to multiple or novel environmental changes, such as those driven by anthropogenic activity. I use the 2-spot ladybird intraguild predation system, where resources and predation risk are highly variable, to explore the role of maternal effects in the response of a native species to an invasive predator, as well as answering outstanding questions about how maternal effects function under complex and antagonistic sets of variables. The results indicate that it is unlikely that maternally mediated changes in egg phenotype will improve the survival of 2-spot ladybird offspring in the face of predation from larvae of the invasive harlequin ladybird. They do, however, demonstrate the importance of studying maternal effects in the context of the multiple environmental factors, which more accurately represent the complex environments in which organisms live and evolve, corroborating recent theoretical predictions. Finally I provide evidence of the multifaceted nature of parental effects in aposematic species and reveal the role that they may play in shaping the variation in defence and warning coloration observed in adult populations.

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CHAPTER 1

INTRODUCTION

"The only way to make sense out of change is to plunge into it, move with it, and join the dance"

- Alan Watts

Across all environments change is the one true constant. Whether it be decadal, annual, seasonal or at time scales of minutes or hours, both abiotic and biotic components of environments are in a constant state of flux. Such change can be 'natural' but also, and recently more commonly, it can be driven by anthropogenic activity (Halpern *et al.* 2008; Sutherland *et al.* 2016). The pervasive effect of the human 'niche builder' (Laland *et al.* 2000) now extends to every ecosystem on earth (Steffen *et al.* 2007) and is influencing not only the specific nature of changes experienced by organisms, e.g. new and exotic invasive species (Mack *et al.* 2000), but also the rate of change, e.g. rapidly increasing global temperatures (IPCC 2013). As well as needing to understand the threats that these changes pose to our own and other species, the 'Anthropocene' is providing us with a unique opportunity to study how organisms adapt to novel changes in their environments (Visser 2008). For example fluctuating environmental conditions are associated with adaptive reproductive strategies, known as maternal effects, which partition maternal resources in a way which maximises reproductive success in specific offspring

environments (Uller 2008). Their consequent role in determining key life-history parameters means that they have a significant influence on population dynamics and the rate and direction of evolution (Raesaenen & Kruuk 2007; Day & Bonduriansky 2011). It is therefore critically important to study maternal effects in the context of current and future environmental change, if we are to fully understand the response of organisms to the challenges thrown up by the Anthropocene. This aspect of transgenerational inheritance, however, remains poorly explored (Cartwright *et al.* 2014). This thesis investigates the role of maternal effects in the response of a chemically defended and warningly coloured (aposematic) ladybird to environmental change, specifically the presence of a novel and invasive predator species.

What is a maternal effect?

Falling under the general umbrella of early life effects, where conditions at the beginning of an organisms life cycle can have profound consequences for its adult phenotype (Figure 1.), the term 'maternal effects' has been used throughout the literature to refer to a number of similar but qualitatively different phenomena (Wolf & Wade 2009; Day & Bonduriansky 2011). Here and throughout this thesis I use the term 'maternal effects' to describe the alteration of offspring phenotype, via the maternal phenotype, independent of genetic inheritance (nuclear, mitochondrial and in the case of plants chloroplast; Mousseau & Fox 1998). The mechanisms underlying maternal effects include, but are not limited to, epigenetic inheritance (e.g. DNA methylation states (Weaver *et al.* 2004)), the transfer of hormones (Groothuis *et al.* 2005), macronutrients (Royle *et al.* 1999) and micronutrients (e.g.

compounds with antioxidant capacities (Blount *et al.* 2000)), antibodies (Boots & Roberts 2012), and defence chemicals (Winters *et al.* 2014b).

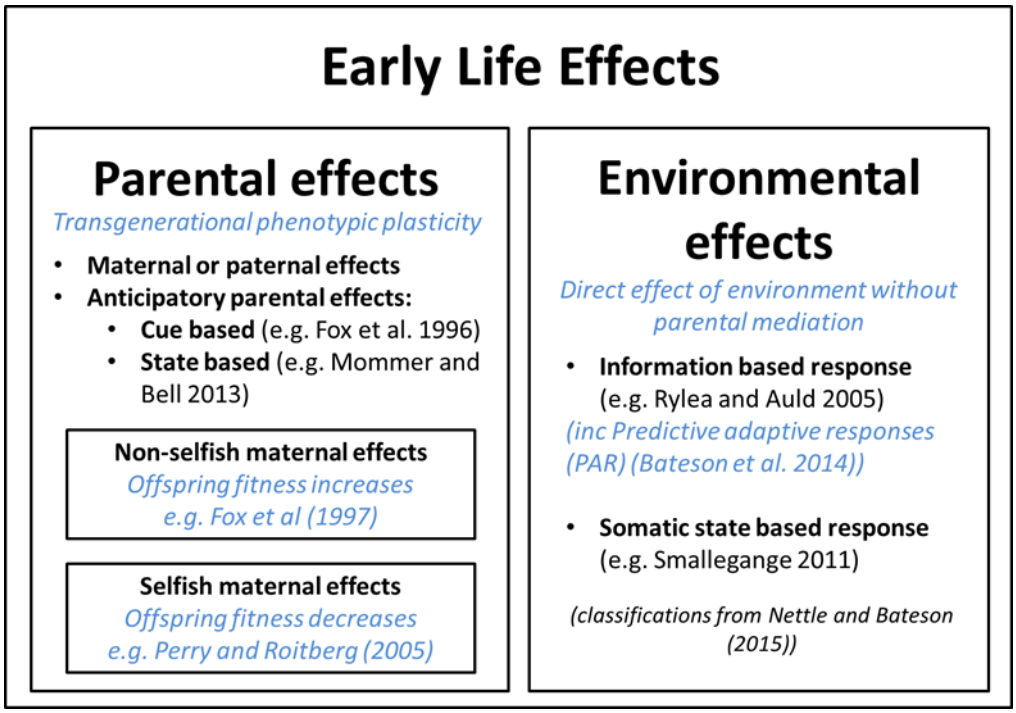

Figure 1. Schematic illustrating how maternal effects and the different classifications of maternal effects fit into the larger theme of early life effects

Maternal effects can occur pre- and post-egg laying (oviposition) or birth (e.g. Weaver *et al.* 2004; Bestion *et al.* 2014), and for those species that exhibit no parental care they can be a powerful mechanism of adapting offspring phenotype to the future environment (Mousseau & Fox 1998). Such transgenerational phenotypic plasticity occurs in response to reliable signals of the future offspring environment and is specifically referred to as an anticipatory maternal effect (AME) (Figure 1; Marshall & Uller 2007). AMEs enable mothers to dynamically adjust the classic offspring number – offspring 'quality' reproductive life-history trade-off (Smith & Fretwell 1974) in order to maximise the total number of surviving offspring and

therefore their own fitness (Uller 2012). This can involve, for example, the alteration of offspring phenotype to maximise survival in the face of environmental change (Marshall & Uller 2007). A classic example of such 'phenotypic matching' is found in the seed beetle (*Stator limbatus*) which lays fewer, larger eggs on tougher seeds, which then lead to larger larvae that are able to penetrate the seed coat (Fox *et al.* 1997a). Females of this species also lay smaller numbers of 'shielded' eggs, a costly but effective egg defence against parasitism, in the presence of parasitic wasps, thus decreasing egg parasitism risk (Deas & Hunter 2013).

Anticipatory maternal effects can be state based or cue based. State based AMEs involve a change in maternal state in response to an environmental change that also correlates with the future offspring environment, which then in turn alters offspring phenotype. For example, gravid females of numerous species produce offspring with morphological or behavioural adaptations that maximise survival in the face of predators after exposure to either predators or predator scent (Agrawal *et al.* 1999; Storm & Lima 2010; Coslovsky & Richner 2011; Bestion *et al.* 2014). In all cases the predators are predators of the adults as well as offspring and therefore maternal state will be affected as she responds to the elevated mortality risk, either perceived or actual. In contrast, cue based AMEs involve the response of females to a cue which reliably indicates the nature of the offspring environment, independently of state e.g. exposure to predators that pose a risk to offspring but not to the mothers themselves (Morosinotto *et al.* 2013; Stratmann & Taborsky 2014).

The distinction between cue based and state based anticipatory maternal effects is not one that is emphasised in the literature, but is worth highlighting. This is because, while in each case the changes in offspring phenotype match the criteria required for AMEs, the underlying mechanisms are likely to be different. Where the

predator is a risk to both mothers and offspring the consequent alteration seen in offspring phenotype may result from the physiological effect of the mother being in a high predation or 'stressful' environment (Travers *et al.* 2010). In such circumstances it is imperative to establish that any changes in offspring phenotype actually increase the number of surviving offspring (Burgess & Marshall 2014); i.e. they are adaptive opposed to merely representing the sublethal effects of predator induced maternal stress on fecundity (Sheriff *et al.* 2009; Wells 2011, 2012). It is therefore crucially important in each case to establish whether maternal fitness is actually increased by the change in offspring phenotype before it can be established to be an adaptive response (Marshall & Uller 2007).

As stated above, maternal effects are predicted to be selected for when they enhance maternal fitness (Wolf & Wade 2009). Somewhat counterintuitively, however, this does not necessarily require an increase in individual offspring fitness (Marshall & Uller 2007). For example, some mothers manipulate levels of cannibalism in their offspring, in response to resource limitation (Perry & Roitberg 2005a; Wong *et al.* 2014). Under resource-poor conditions the number of surviving offspring is maximised by cannibalistic behaviour, but the fitness of cannibalised offspring is lower than that of their sibling cannibals, despite the inclusive fitness benefits (Parker & Smith 1990). Overall, therefore, when resources are scarce an alteration in offspring phenotype which encourages cannibalism can result in maternal fitness benefits, at the expense of some offspring. Other examples of such selfish maternal effects include oviposition (egg laying) site choice in a number of insects. In egg laying species both where and how (i.e. cluster size) eggs are laid are considered to be critical components of the offspring phenotype (Roitberg 1998; Mitchell *et al.* 2013; Reedy *et al.* 2013). For example, the hoverflies *Episyrphus*

balteatus and *Syrphus ribesii* oviposit eggs in suboptimal environments (e.g. higher predation risk or lower resource availability) when time-limited, in order to avoid the risk of failing to lay all of their eggs (Sadeghi & Gilbert 2000). Though those individual offspring that are laid in low quality environments will not have as high a fitness as if they had been laid in a better quality environment, overall the female will have maximised the number of offspring laid across different environments, thereby maximising the number of surviving offspring and her fitness (Mangel, 1989). In summary, maternal fitness benefits are accrued through non selfish AMEs via an increase in the total surviving offspring via increases in offspring fitness. In contrast for selfish AMEs, they are accrued at the expense of individual offspring fitness, as this strategy increases the overall number of surviving offspring (Sevenster *et al.* 1998; Rosenheim 1999).

Theoretical predictions and empirical findings for AMEs

Theoretical models predict that different types of maternal effects can develop depending on the rate and direction of environmental change (Hoyle & Ezard 2012). Anticipatory maternal effects are expected to evolve under conditions where the environment fluctuates predictably between generations and where the phenotypic traits in question are under strong selection (Kuijper & Hoyle 2015), e.g. ones linked to key life-history parameters such as size (Kuijper & Johnstone 2013). A number of benchmark studies clearly identifying AMEs in plant and animal species (Fox *et al.* 1997a; Agrawal *et al.* 1999; Galloway & Etterson 2007; Walsh *et al.* 2015) highlight the importance of reliable environmental cues indicating the future offspring environment in the evolution of AMEs. In all of these studies the mother is exposed

to a cue which directly links to the presence of a predator or an abiotic change in the offspring environment, e.g. maternal *Daphnia ambigua* exposed to predator kairomones (chemical cues) (Walsh *et al.* 2015) or *Campanulastrum americanum* plants exposed to differing light conditions (Galloway & Etterson 2007). In fact it is a lack of this critical requirement in many systems that have previously been used to investigate AMEs, that Uller and colleagues argue contributed to the overall weak evidence found for AMEs in a recent meta-analysis (Uller *et al.* 2013). It is therefore conceivable that, if such conditions are satisfied, maternal effects could facilitate species response and adaptation to the rapid change expected, and in some environments already currently being experienced, due to anthropogenic activity (Monaghan 2008; Hoyle & Ezard 2012). We investigate the role of AMEs in determining the reproductive response of females to an invasive offspring predator, which produces similar cues to a familiar predator in Chapter 2.

Multiple environmental variables and AMEs

Both the theoretical exploration and empirical tests of maternal effects, and for that matter AMEs, focus predominantly on response to univariate environmental change (Townley & Ezard 2013; Bestion *et al.* 2014; Kuijper *et al.* 2014; Shama *et al.* 2014). Although this work offers vital insights into the nature of AMEs it does not encompass the full complexity of the offspring environment. Multiple factors impact offspring fitness and survival (Stearns 1992), and the optimal phenotype under environmental conditions dominated by change in one factor may be strikingly different to the optimal phenotype dictated by another factor (Relyea & Hoverman 2003). Under these circumstances when such different environmental variables covary, they can place antagonistic selection pressures on offspring phenotype. To draw on an example from intragenerational opposed to intergenerational plasticity,

tadpoles of the wood frog (*Rana sylvatica*) develop short bodies with small mouths and deep tails in response to increased predation risk, but the reverse morphological characteristics develop in response to increased competition (Relyea & Auld 2005). The consequent phenotypes shown by tadpoles under crossed gradients of predation risk and conspecific density demonstrate a balance between the two phenotypic optima, with the response to predation risk being highest in low competition environments and vice versa (Relyea 2004). One would predict similar responses in offspring characteristics influenced by maternal effects, including AMEs; we test this prediction for the first time in Chapter 3.

Aposematism and AMEs

It is also important to consider that many offspring traits are intrinsically linked and therefore maternal effects are likely to be multivariate (Kuijper *et al.* 2014). Such offspring traits include those involved in aposematism, where individuals advertise toxic or distasteful secondary chemical defences via conspicuous colouration (Poulton, 1890). Aposematism has been identified in wide variety of organisms including frogs (Summers & Clough 2001), beetles (Blount *et al.* 2012), moths (Nokelainen *et al.* 2012), and marine slugs (Nudibranchs; Cortesi & Cheney 2010) and maternal effects are hypothesised to have been important in its evolution (Brodie & Agrawal 2001). Females of chemically defended species are known to provide their offspring with defensive compounds that offspring may not immediately be able to sequester from their environment (Hutchinson *et al.* 2008; Itoi *et al.* 2014), the possession of which increases offspring survival (Stynoski *et al.* 2014). Maternal allocation of both defence and signalling compounds has also been recorded in the aposematic ladybird *Coccinella septempunctata*, with offspring defence and colour being a good predictor of adult defence and colour at eclosion (Winters et al., 2014).

It is therefore apparent that maternal investment can influence defence levels and warning colouration in offspring. This is particularly important considering that early life stages are often the most vulnerable to predation (Stearns, 1992), however, as outlined above, the risk of offspring predation is likely to vary between reproductive bouts. As aposematic defences incur costs (Holloway *et al.* 1991a; Zagrobelny *et al.* 2007) there is likely to be a trade-off between the number of offspring and their defence level (Smith & Fretwell 1974). Mothers are therefore likely to benefit from investing in offspring defence levels in an environmentally dependent way.

AMEs have been demonstrated in relation to offspring colour in the predatory stink bug *Podisus maculiventris*, which produces darker eggs when laying on top of leaves than when laying underneath leaves, purportedly to protect eggs from harmful UV exposure (Abram *et al.* 2015). Whether such maternal control of colour exists in aposematic species is however underexplored, as is the role of mothers in determining offspring signalling honesty under different environmental conditions. Signalling honesty in aposematic organisms involves a positive correlation between the toxic defence and strength of the colourful warning signal, e.g. its conspicuousness (Ruxton *et al.* 2004). Multiple theories exist which describe the possible mechanisms by which such signalling honesty is maintained in the wild (reviewed in, Summers *et al.* 2015), however though such honesty is found in across multiple populations and species (Cortesi & Cheney 2010; Santos & Cannatella 2011; Manuel Vidal-Cordero *et al.* 2012; Arenas *et al.* 2015a) it is not universal (Darst *et al.* 2006; Wang 2011). For example, Blount et al. (2012) demonstrated that a positive correlation between carotenoid and coccinelline levels existed in female 7-spot ladybirds (*Coccinella septempunctata*) across treatments but there was a negative correlation between the two in males. We investigate whether aposematic

mothers can manipulate offspring signals and signalling honesty in response to reliable cues in Chapters 4 and 5.

Parental phenotype and AMEs

By its very definition an intrinsic component of AMEs is the maternal phenotype, however this in turn is also highly variable and may influence the extent to which a particular AME is expressed. For example, the female water strider (*Aquarius paludum insularis*) increases the depth at which she lays eggs in response to egg parasitism risk. However, there are physiological costs to diving (Hirayama & Kasuya 2014). A females' ability to dive is therefore likely to vary depending on her phenotype and therefore so will the extent of her response to parasite presence when laying eggs, i.e. the AME. Such differences in the expression of AMEs between different maternal phenotypes may be particularly prevalent in those species with extreme phenotypic differences or morphs, e.g. females of different colours or sizes (Darst et al. 2006; Cortesi & Cheney 2010), though further investigation is needed to verify this assertion.

Female reproductive investment is also strongly influenced by the quality of the male with which she mates, a type of AME known as differential allocation (DA; Ratikainen & Kokko 2010). Such signals of male quality are predominantly either visual, for example ornamentation in birds (Andersson & Iwasa 1996), or chemical, e.g. pheromones (Johansson & Jones 2007). Mothers can either increase ('positive DA' (e.g. Sheldon 2000; Horvathova *et al.* 2012)) or decrease ('negative DA' or 'reproductive compensation' (e.g. Saino *et al.* 2002; Bolund *et al.* 2009)) their investment in offspring, in response to male phenotype or 'attractiveness'

(Ratikainen & Kokko 2010). For example female mallards (*Anas platyrhynchos*) lay larger eggs with higher albumen lysozyme concentration after mating with more attractive males (Cunningham & Russell 2000; Giraudeau *et al.* 2011). As with all AMEs the alteration of offspring phenotype brought about in response to male quality also varies depending on maternal phenotype. Returning to the mallards for an example, the strength of maternal effect stimulated via male quality is dependent on female age and experience. Older females paired to larger males had both higher nesting success and brood survival, however the nesting success of younger females was only improved when they mated with males with more colourful plumage and there was no effect of any male condition indices on the survival of their broods (Sheppard *et al.* 2013). In summary, the interaction of female phenotype and both environmental variables and male quality may influence the nature of AMEs. However, little is known about how female response to male quality and variation in female phenotype itself, interact with predictable environmental variation to determine offspring phenotype via maternal effects (Bonduriansky & Head 2007). Further work combining these variables to investigate maternal effects, including AMEs, is therefore likely to give a more refined and holistic picture of the role of transgenerational effects in determining offspring phenotype in complex and variable environments.

Ladybird system

The aposematic two-spot ladybird, *Adalia bipunctata*, is native to the UK and an ideal species with which to answer questions on reproductive investment under varying environmental conditions as outlined above. Females lay multiple batches of bright

yellow-orange eggs (Figure 2), either singly or in clutches, on aphid infested plants (Seagraves 2009). *A.bipunctata* adults and larvae feed on aphids, the colonies of which are a highly ephemeral resource, patchily distributed throughout the environment (Van Emden & Harrington 2007), with different species of aphid also varying dramatically in their nutritional suitability (Zhang *et al.* 2012). Resource availability and quality therefore varies dramatically both spatially and temporally throughout each females' laying period (3-6 months; Hodek *et al.* 2012).

Figure 2. Cluster of *A. bipunctata* eggs laid on underside of leaf of *Vicia faba* bean plant infested with aphid *Acyrthosiphon pisum*

Aphid colonies also attract a number of additional predators, including other ladybird species (Phoofolo & Obrycki 1998), the adults and larvae of which will also predate ladybird eggs, a phenomenon known as intraguild predation (Polis *et al.* 1989). The extent of such predation is dependent on the nature of ladybird egg chemical defence, both quantitative and qualitative (Hemptinne *et al.* 2000a; Kajita *et al.* 2010). Eggs, like the adults that produce them, are protected by species-specific alkaloids and the tolerance of these toxins varies between ladybird species (Appendix I.). This differential tolerance leads to many asymmetrical intraguild interactions. For example, *A. bipunctata* larvae find eggs of the seven-spot ladybird (*Coccinella septempunctata)* unpalatable and those that are consumed have a lethal

effect (Hemptinne *et al.* 2000a), however the reverse is true of predation of *A.bipunctata* eggs by *C.septempunctata* larvae (Sato & Dixon 2004). Larvae of the invasive harlequin ladybird (*Harmonia axyridis)* are particularly voracious and have a high tolerance for heterospecific alkaloids (Katsanis *et al.* 2013), which is thought to have contributed to their success as invaders and the decline of many native species within their invasive range (Roy *et al.* 2012; Comont *et al.* 2014).

Offspring predation

In ladybirds the risk of egg predation, as with the early life stages of most animals, is high, however females are able to detect and respond to this risk, an adaptation that is particularly important for a species with no maternal care (Perry & Roitberg 2005b; Kajita *et al.* 2006; Kajita *et al.* 2009; Mishra *et al.* 2012). A mixture of species-specific low volatility chemicals (Magro *et al.* 2010), predominantly alkanes (Hemptinne *et al.* 2001; Magro *et al.* 2007), originating from the anal disc of predatory larvae (Laubertie *et al.* 2006) are used as a reliable cue of offspring predation risk by laying females ('larval tracks'; Ruzicka 2006; Magro *et al.* 2007). As with intraguild predation, female response varies between the species laying and the larval species detected (Appendix II.), but generally females are initially deterred from laying eggs on detection of these 'larval tracks'. The strength of this AME is density-dependent (Oliver *et al.* 2006; Mishra *et al.* 2012), and is stronger in response to tracks of related larvae (Martini *et al.* 2013) as well as decreasing with increasing age and experience of ovipositing females (Frechette 2004). Additionally females respond to tracks up to a month in age, though signal longevity varies between species of larvae (Hemptinne *et al.* 2001; Ruzicka 2002; Oliver *et al.* 2006; Ruzicka 2006). However, as stated above, for ladybirds resource and risk are frequently correlated, so avoidance of predation risk comes at the cost of offspring food provision and so may

not always be advantageous. To this end females also increase the size of clusters in response to both conspecific and heterospecific tracks (Ruzicka, 2006), an adaptation already experimentally demonstrated to reduce predation (Agarwala and Dixon, 1993). Toxin level is also known to vary between eggs laid by the same female (Kajita *et al.* 2010), though the relationship between egg toxin level and measures of colouration indicate that in the absence of predators egg conspicuousness is an 'honest' signal of toxin level (Winters et al., 2014). However, whether an individual female modulates toxin investment in offspring (eggs) and how the relationship between toxin level and colour may change in response to predation risk remains unknown. This is tested in Chapters 2 and 5.

Offspring resources

It is not just the extent of offspring risk that females assess when laying, but also resource quality and abundance, i.e. the size, suitability and developmental stage of an aphid colony (Seagraves 2009). As previously mentioned aphid colonies are ephemeral and patchily distributed throughout the environment and females are known to adjust laying behaviour in response to reliable chemical cues of the offspring trophic environment, e.g. cluster size (Dixon & Guo 1993). It is also worth noting at this point that sibling cannibalism amongst ladybird larvae, including *A.bipunctata*, is frequent and is thought to be an adaptive strategy under resource-poor conditions, where it maximises the number of surviving offspring, and therefore maternal fitness (Pfennig 1997). The consumption of conspecific eggs by larvae has no adverse effect on development and in some cases such cannibalistic behaviour has been shown to be more beneficial than consuming aphids (Agarwala & Dixon 1992; Osawa 2002). This is unsurprising as conspecifics have the appropriate metabolic pathways for detoxifying conspecific defensive chemicals, and eggs are

highly nutritious (Sloggett & Davis 2010). It has also been postulated that cannibalistic larvae can sequester conspecific alkaloids (Kajita *et al.* 2010), meaning that they gain both nutritional and defence benefits. In *H.axyridis,* mothers manipulate the extent of cannibalistic behaviour via AMEs which involve the laying of trophic eggs in response to low resource availability (Perry & Roitberg 2005b, 2006). Higher egg toxin levels and larger cluster sizes have also been linked to an increasing risk of sibling cannibalism (Agarwala & Dixon 1992; Roy *et al.* 2007; Kajita *et al.* 2010) and this leads to an interesting potential conflict in the response of females to the correlated environmental variables of predation risk and resource. The offspring phenotype (high egg toxicity and large clusters) that is most beneficial under high predation risk is a phenotype that potentially also increases the risk of cannibalism, which is less beneficial to maternal fitness when resources are abundant. We investigate this apparent paradoxical situation in Chapter 3, where we identify how AMEs are determined under multiple covarying environmental selection pressures.

Parental investment

Like many ladybirds reproductive investment in *A.bipunctata* follows a triangular fecundity function whereby the number of eggs produced increases after eclosion to a peak at around 30 days, depending on diet and time of first mating, and then declines (Hodek *et al.* 2012). The size of clusters laid by female ladybirds also increases with female size (Dixon & Guo 1993; Ware *et al.* 2008). Though intraspecific variation in egg size does occur, relationships between egg size and clutch size, illustrating the classic offspring number-offspring size trade-off (Smith &

Fretwell 1974) have only been identified at the interspecific as opposed to intraspecific level (Stewart *et al.* 1991a; Stewart *et al.* 1991b). It is postulated that trade-offs at the intraspecific level may be mediated by egg composition (Sloggett & Lorenz 2008), for example through costly toxin investment (Holloway *et al.* 1991a; Zagrobelny *et al.* 2007), though this remains to be investigated.

Female ladybirds also show mate choice, for example melanic morphs are preferentially chosen over other morphs in a number of species (Hodek & Ceryngier 2000), including *A.bipunctata* (Majerus *et al.* 1982a; Majerus *et al.* 1982b). Additionally females of the ladybird *Propylea dissecta* that mate with melanic males have higher egg viability than when mating with a light morph male (Mishra & Omkar 2014). Clearly, females can discriminate between different male morphs and this differentiation influences their reproductive investment and success. However, the extent to which fine scale variation in aspects of male quality, e.g. conspicuousness (Maan & Cummings 2008), influences female investment is unknown. AMEs may also differ depending on female morph. As with any reproductive investment, the direction and strength of anticipatory maternal effects is known to be influenced by the potential costs as well as the benefits to each female (Godfray 1995; Uller 2012). For example, female grass miners (*Chromatomyia nigra)* oviposit eggs in the vicinity of their foraging sites, however site choice is dictated predominantly by site suitability for adults as opposed to offspring foraging (Scheirs *et al.* 2000). As would be expected, female condition also heavily influences maternal response to such trade-offs and therefore female reproductive investment, including maternal effects (Stearns 1992). Offspring of the northern leopard frog (*Lithobates pipiens)*, for example, respond to predator presence through plastic changes in body size, but this change is more pronounced in offspring laid by females in better condition

(Bennett & Murray 2014). Melanic morphs of aposematic species, as in *A.bipunctata* populations, have thermoregulatory advantages (DeJong *et al.* 1996; Hegna *et al.* 2013), but face costs of increased predation risk in comparison to non-melanics (Arenas *et al.* 2015b). There are also differences in the physiology of melanic and non-melanic morphs, for instance in their immune defence (Dubovskiy *et al.* 2013) and in aposematic species in their levels of chemical defence (Bezzerides *et al.* 2007). One may therefore also expect anticipatory maternal effects to differ to some extent between morphs, in a way which reflects the differential costs and constraints associated with each phenotype and therefore there also to be differences in survival between offspring in different environments. This has, however, yet to be tested but is particularly important when considering the role of AMEs in response to anthropogenic change, which if they mediate differences in offspring survival may contribute to a shift in the balance of morphs within a population.

In summary, *A.bipunctata* reproduce in a 'ladybird-eat-ladybird' world, where resources are variable, but where they can detect reliable cues about the nature of the future offspring environment. I therefore use this system to explore the role of cue based AMEs in response to an invasive predatory species, as well as answering some outstanding questions about how AMEs function under complex sets of variables. Specifically I address the following questions:

Chapter 2) Do female *A.bipunctata* modify offspring phenotype, including egg toxin level, in response to the presence of an invasive offspring predator? Is there a trade-off between egg number and egg toxin level?

Chapter 3) How do *A.bipunctata* modify offspring phenotype in response to antagonistic selection pressures?

Chapter 4) How does maternal phenotypic variation in *A.bipunctata*, in this case female morph, lead to variation in offspring aposematic phenotype?

Chapter 5) How does maternal and paternal phenotypic variation influence offspring phenotype in an aposematic species under the risk of predation and risk of cannibalism?

CHAPTER 2

Reproduction in Risky Environments: the Role of Invasive Egg Predators in Ladybird Laying Strategies

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Abstract

Reproductive environments are variable and the resources available for reproduction are finite. If reliable cues about the environment exist, mothers can alter offspring phenotype in a way that increases both offspring and maternal fitness ('anticipatory maternal effects' - AMEs). Strategic use of AMEs is likely to be important in chemically defended species, where the risk of offspring predation may be modulated by maternal investment in offspring toxin level, albeit at some cost to mothers. Whether mothers adjust offspring toxin levels in response to variation in predation risk is, however, unknown, but is likely to be important when assessing the response of chemically defended species to the recent and pervasive changes in the global predator landscape, driven by the spread of invasive species. Using the chemically defended two-spot ladybird, *Adalia bipunctata*, we investigated

reproductive investment, including egg toxin level, under conditions that varied in the degree of simulated offspring predation risk from larval harlequin ladybirds, *Harmonia axyridis*. *H. axyridis* is a highly voracious alien invasive species in the UK and a significant intraguild predator of *A. bipunctata*. Females laid fewer, larger egg clusters, under conditions of simulated predation risk (P+) than when predator cues were absent (P-), but there was no difference in toxin level between the two treatments. Among P- females, when mean cluster size increased there were concomitant increases in both the mass and toxin concentration of eggs, however when P+ females increased cluster size there was no corresponding increase in egg toxin level. We conclude that, in the face of offspring predation risk, females either withheld toxins or were physiologically constrained, leading to a trade-off between cluster size and egg toxin level. Our results provide the first demonstration that the risk of offspring predation by a novel invasive predator can influence maternal investment in toxins within their offspring.

Introduction

Maternal fitness is increased by maximising the number of offspring that survive to reproduce (Roff 1992; Stearns 1992). As the resources available for reproduction are finite, there is a trade-off between fecundity and per-offspring maternal investment (Lack 1947a; Smith & Fretwell 1974). Classically egg size has been used to identify this trade-off (Smith *et al.* 1989; Berrigan 1991; Riesch *et al.* 2012), however, while egg size may often be a good proxy for maternal investment, there are exceptions (Kaplan 1992; Marshall & Bolton 2007). In some cases measuring components of egg composition, e.g. hormones, carotenoids or other micronutrients that may influence offspring fitness (Veiga *et al.* 2004), can be a more accurate representation of per-offspring maternal investment (Nager *et al.* 2000; Blount *et al.* 2004). Egg chemical defence is one such component that can influence offspring survival (Itoi *et al.* 2014), and is particularly important in species with no or little parental care, such as many insect species (Blum & Hilker 2002). However, it can be costly (Higginson *et al.* 2011), both metabolically, with costs associated with toxin production and storage (Blount *et al.* 2009; Blount *et al.* 2012), and if sequestered from the environment, where costs are associated with foraging for the toxins themselves (Kojima & Mori 2015). Therefore, trade-offs may exist between egg toxin level and the size and number of offspring, but this remains to be tested.

Optimal per-offspring investment is also dependent on the reproductive environment; that is the quality of the environment into which the offspring will emerge (Bernardo 1996b). To maximise offspring survival in poorer quality environments, the optimal investment will be larger than in environments with more favourable conditions (McGinley *et al.* 1987; Einum & Fleming 1999). Where reliable cues about the nature of the offspring environment exist, mothers can adjust offspring phenotype in order to

maximize offspring survival. Such ‘Anticipatory Maternal Effects’ [hereafter AMEs; (Burgess & Marshall 2014)] involve an increase in maternal fitness through a concomitant increase in offspring fitness (Marshall & Uller 2007; Burton & Metcalfe 2014) and examples of predator-driven AMEs have been identified across multiple taxa (Storm & Lima 2010; Coslovsky & Richner 2011; Giesing *et al.* 2011; Roche *et al.* 2012; Bestion *et al.* 2014). For selection to favour AMEs, the maternal environment at the time of reproduction must be a good predictor of the environment that her offspring will experience, and the cost of plasticity must be outweighed by the increase in maternal fitness accrued through the change in offspring phenotype (Marshall & Uller 2007).

Studies of AMEs, and of maternal effects in general, focus heavily on natural environmental variation, for instance fluctuations in food abundance and the aforementioned predation risk (Marshall & Uller 2007; Bonduriansky & Day 2009). This makes sense as it is adaptations to these natural environmental fluctuations and perturbations that will have been selected for over the course of a species evolutionary history (DeWitt & Scheiner 2004). However, modern day ecosystems are currently experiencing dramatic, anthropogenically driven change, for example from pollution, land use change, pesticide use, invasive species and climate change (Halpern *et al.* 2008; Ellis 2011). Maternal effects are a powerful mechanism by which females can respond to this change and consequently should be considered when assessing the impact of any of these anthropogenically driven factors on species and populations. For instance the priming of offspring phenotype to increases in temperature, drought and heavy metal abundance, via maternal exposure to these factors has been demonstrated in plants and a species of butterfly (Fischer *et al.* 2003; Ou *et al.* 2012; Suter & Widmer 2013). Furthermore alterations

in the maternal environment, induced by anthropogenic change, may also have indirect beneficial effects on offspring fitness, again mediated by maternal effects. One such case is found in *Daphnia magna* where offspring produced by mothers reared at higher temperatures had lower susceptibility to disease than offspring of control mothers (Garbutt *et al.* 2014).Unlike pollutants and climate change there has been little focus on the way maternal effects may mediate the impact of invasive species on natives. This is surprising considering the increase in the number and global spread of invasive species in recent decades (Sax *et al.* 2007; Kenis *et al.* 2009), and their well documented negative impact on the fitness of native species, e.g. via predation of offspring (Mooney & Cleland 2001). Consequently, determining how females modulate investment, via maternal effects such as AMEs, in the face of such novel offspring predators, is crucial in order to understand the complex effects of invasives on native species.

Conspicuous and chemically defended (aposematic) ladybirds are ideal species in which to investigate the reproductive strategies of females in environments with variable levels of offspring predation risk, by an invasive predator. Such ladybirds show no maternal care and lay clusters of brightly coloured eggs that are chemically defended by autogenously produced alkaloids (Daloze *et al.* 1995); known to be a costly form of defence in adults (Holloway *et al.* 1991b). These alkaloids are present in the tissue and on the surface of ladybird eggs (Hemptinne *et al.* 2000b; Omkar *et al.* 2004), and (between- and within-maternal) variation in egg alkaloid levels affects egg predation rates (Kajita *et al.* 2010). The eggs have numerous predators (Hemptinne *et al.* 2012; Smith & Gardiner 2013a), including the larvae of invasive ladybird species (Ware & Majerus 2008; Gardiner *et al.* 2009; Katsanis *et al.* 2013). The presence and abundance of such predators varies greatly in space and time

(Ingels & De Clercq 2011; Smith & Gardiner 2013b), meaning that optimal toxin investment can vary between reproductive environments. Females delay the onset of egg laying and lay fewer eggs in response to chemical cues that reliably indicate the presence and abundance of larval predators (Doumbia *et al.* 1998; Yasuda *et al.* 2000; Ruzicka 2001; Frechette *et al.* 2004). Furthermore, egg clustering deters predation by heterospecific larvae (Agarwala & Dixon 1993). However, whether females modulate toxin investment in eggs, considering the high potential costs of toxin production, or cluster size in response to predation risk remains unknown.

We investigated the effects of simulated predation risk on the egg laying behaviour of ladybirds including their investment in egg toxins. Two-spot ladybirds, *Adalia bipunctata*, were allowed to lay eggs in environments that either contained larval tracks of harlequin ladybirds, *Harmonia axyridis*, (P+) or that contained no tracks as a control (P-). *H. axyridis* is an invasive species in the UK, and being highly polyphageous and competitive, it poses a serious risk to *A. bipunctata* populations in the wild (Brown *et al.* 2008; Roy *et al.* 2012). Eggs of *A. bipunctata* contain the alkaloid adaline (Holloway *et al.* 1991a) and we predicted that females in P+ conditions would lay eggs that contained higher adaline concentrations compared to females in P-, control, conditions and that consequently there would be a trade-off between egg number and egg toxin level. As egg clustering deters predation by heterospecific ladybird larvae (Agarwala & Dixon 1993), we also predicted that larger individual clusters of eggs would be laid under P+ conditions than under P- conditions. Finally we predicted that P+ females would delay egg laying (increased latency) and produce fewer eggs overall than P- females, in agreement with previous studies (Doumbia *et al.* 1998; Hemptinne *et al.* 2001; Agarwala *et al.* 2003).

Materials and Methods

A stock culture of *A. bipunctata* (f. *typica*), obtained from Syngenta Bioline (Little Clacton, Essex, CO16 9QG), was maintained in a cage on an *ad lib*. diet of pea aphids, *Acyrthosiphon pisum*, at 20^{o}C in a 16L:8D h photoperiod. The *A. pisum* prey were reared in cages on dwarf bean (*Vicia faba*) under the same abiotic conditions as the *A. bipunctata*. Experimental individuals of *A. bipunctata* were 1st generation virgin adults of known age (20-25 days post eclosion) reared from individuals obtained from the stock culture: 44 females and 44 males from five different adult pairs. Each female was mated with a non-sibling male and after 1h females were removed and placed individually into an experimental Petri dish that differed in simulated predation risk (see below) and provided with *A. pisum ab lib*. Females from different sibling clusters were distributed evenly between the two treatment levels, so that family ID and mate ID were represented equally in both P+ and P- treatments. Family ID refers to the adult pair from which the experimental females were reared i.e. the identity of their parents, and mate ID to the identity of the parents (i.e. adult pair) from which experimental males were reared. Experiments were carried out in an incubator (Percival® model I-41LL, 505 Research Drive, Perry, IA 50220 USA) at 18^{o}C and a 16L:8D h photoperiod.

To create an environment that conferred a simulated risk of predation (P+), 4th instar *H. axyridis* larvae were placed, without food, into individual sterile Petri dishes (9cm diam.), each containing a semicircle of corrugated filter paper (9cm diam.) and left for 24 h (Doumbia *et al.* 1998; Magro *et al.* 2007), after which time they were removed. A control environment of no simulated predation risk (P-) consisted of a sterile Petri dish (9cm diam.) and a clean semicircle of corrugated filter paper that had not been in contact with *H. axyridis*. Mated *A. bipunctata* females were placed

individually into a P+ or P- Petri dish, with adlib *A. pisum* and the number of eggs and individual clusters of eggs laid was recorded at 1, 3, 6, 9, 12 and 24 h intervals. Ad. lib *A. pisum* were provided to reduce the risk of filial cannibalism (Ferrer *et al.* 2011) , additionally dishes were monitored for evidence of cannibalism, easily detected through the presence of egg remains, and females were excluded from the analysis if cannibalism had occurred. After 24 h females were removed and, along with all clusters of eggs laid, frozen at - 80^{o}C prior to chemical analysis. A cluster was classified as a group of two or more eggs, with each egg being in physical contact with at least one other egg in that cluster. Each cluster was frozen individually and, depending on cluster size, one to six eggs were randomly selected from each cluster laid by each of the females, with the exception of one female where only one of the two clusters of eggs laid was analysed. These eggs were weighed to the nearest 0.1µg, individual egg weight is referred to as egg mass from this point onwards, and alkaloid (adaline) levels analysed.

Quantifying levels of adaline

Each egg was weighed to the nearest 0.1µg using an XP6U Ultra-microbalance (Mettler-Toledo) and homogenized using a hand held pestle (Fisherbrand™ Pellet Pestle™ Cordless Motor) for 30 s in 200µl chloroform with an internal standard of 1ng/µl E,Z-4,7 tridecadienyl acetate (Pherobank, 6700 AH Wageningen). Samples were then centrifuged at 17.7 x g for 3 min, and an aliquot (100µl) transferred into an autosampler vial. Similarly for adults, the elytra, which unlike the body tissue are purely structural (keratinous) and contain no, or undetectable levels of alkaloids (Holloway *et al.* 1991a; Laurent *et al.* 2002), were removed and the body was weighed to the nearest 0.01mg using an analytical balance (GR-200 A&D® Gemini™) before being homogenised for 60 seconds in 500µl chloroform with an

internal standard of 1ng/ µl E,Z-4,7 tridecadienyl acetate. After homogenization a second 500µl of solvent solution was added. Each sample was then centrifuged at 17.7g and 13.3rpm for 3 minutes. 10µl of extract solution and 90µl of solvent solution was then transferred into an autosampler vial. Samples (2µl) were injected into an Agilent 7890A GC coupled with a 5975B MS fitted with an HP5-ms column (30mx0.25mmx0.25µm film thickness). The injection was in pulsed splitless mode, and the inlet temperature was 250^{o}C. The carrier gas was helium with a flow rate of 1.3 mL/min. The GC temperature programme was 50^{o}C at injection increasing to 140^{o}C at 20^{o}C/min, then from 140^{o}C to 280^{o}C at 5^{o}C/min. Mass spectra operated in SIM mode, scanning for ions m/z (166.2 for Adaline) and (79. 1 for standard). Adaline (ng/mg body tissue) was quantified relative to the internal standard.

Data analyses

All analyses were carried out using R version 3.0.2 (R Development core team 2015). Data were examined for normality, homoscedasticity and outliers. The alpha level was set at 0.05 for all tests and stepwise backwards deletion was employed to reach the minimum adequate model (Crawley 2013). A multinomial logistic regression model (package=mlogit) was fitted to ascertain whether there was a difference in the onset of laying between the two treatments, i.e. if the presence of *H. axyridis* tracks deterred laying.

A general linear model (package=MASS, function=glm) was fitted to the sqrt of total egg number with treatment, total cluster number and female mass (mg) as covariates. Generalized linear modelling (package=MASS, function=glm, family=quasipoisson) was used to identify differences in both the total cluster number and mean size of clusters per female between treatments, with total egg number and

total cluster number as respective covariates and female mass (mg) as a covariate in both models.

There was statistically significant repeatability of egg mass, the weight (mg) of individual eggs, and egg adaline concentration within clusters (Egg adaline: R = 0.749, SE =0.042, CI = 0.656, 0.816, P=0.001; Egg mass: R = 0.599, SE = 0.055, CI = 0.472, 0.69, P =0.001) and females (Egg adaline: R = 0.750, SE = 0.057, CI = 0.609, 0.832, P = 0.001; Egg mass: R = 0.528, SE = 0.068, CI = 0.379, 0.642, P= 0.01). Repeatability was calculated using a generalized linear mixed effects model with a log link for egg adaline and a linear mixed effects model for egg mass in the 'rptR' package following (Nakagawa & Schielzeth 2010). These results supported the use of a subsample of eggs from each cluster as representative of the adaline and mass of eggs per female.

Variation in egg adaline concentration (ng/mg) with treatment, maternal adaline concentration, total egg number or mean cluster size, and a two way interaction between treatment and total egg number/mean cluster size was assessed using generalised mixed effects modelling (package=lme4 (Bates *et al.* 2015)), function= glmer, family=poisson) with female and cluster identity as nested random effects. Variation in egg mass (mg) with treatment, female mass (mg), total egg number or mean cluster size, and a two way interaction between treatment and total egg number/mean cluster size was assessed using linear mixed effects modelling (package=lme4 (Bates *et al.* 2015)), function= lmer) with female and cluster identity as nested random effects. Models were simplified using a backwards stepwise deletion approach (Crawley 2013) and results are reported for all main effects and significant interactions ($P < 0.05$).

There was no difference between the two treatments in whether or not a female cannibalised her eggs (Chi-Sq; X^2_1= 2.530, P=0.112). However, the specific number of eggs cannibalised could not be quantified, and therefore only females that did not cannibalise their eggs were included in the analyses (n(Fem)=28 and n(Cluster)=49).

Results

The latency period before egg laying started did not differ significantly between P- and P+ groups (X^2_1=4.236, P=0.30, R2=0.058; (P-): 17 $\pm$ 2 h, (P+):15 $\pm$ 2 h (mean time till first egg laid $\pm$ SE)). Similarly, the total number of eggs laid by females did not differ significantly between the P- and P+ groups ($F_{1,24}$=0.6965, P=0.413). However, the pattern of laying did differ; in the simulated presence of predators (P+) the total number of clusters laid was significantly smaller (Figure 1 a); $X^2_{1,24}$=7.554, P<0.01), but the mean cluster size was greater (Figure 1 b); $X^2_{1,24}$=4.826, P=0.03) than when predator cues were absent (P-).

Though there was no treatment effect (see above) egg mass, the weight of individual eggs (mg), significantly increased with both mean cluster size (mean cluster size, X^2_1= 4.363, P=0.036; treatment*mean cluster size, NS) and total egg number (total egg number, X^2_1= 3.950, P=0.047; treatment* total egg number, NS).

The concentrations of adaline (mg/ng) in adult females and their eggs were not significantly correlated (X^2_1= 1.044, P=0.307). Adaline levels did not differ significantly between treatments (X^2_1=1.867, P= 0.172) and were not correlated with egg number (total egg number, X^2_1=0.225, P= 0.636; treatment*total egg number, NS). However, there was an interactive effect of treatment and mean cluster size on egg adaline levels (X^2_1=6.428, P= 0.012); there was a positive relationship between

egg adaline concentration and mean cluster size for P- females, whereas the opposite pattern was found for P+ females (Figure 2).

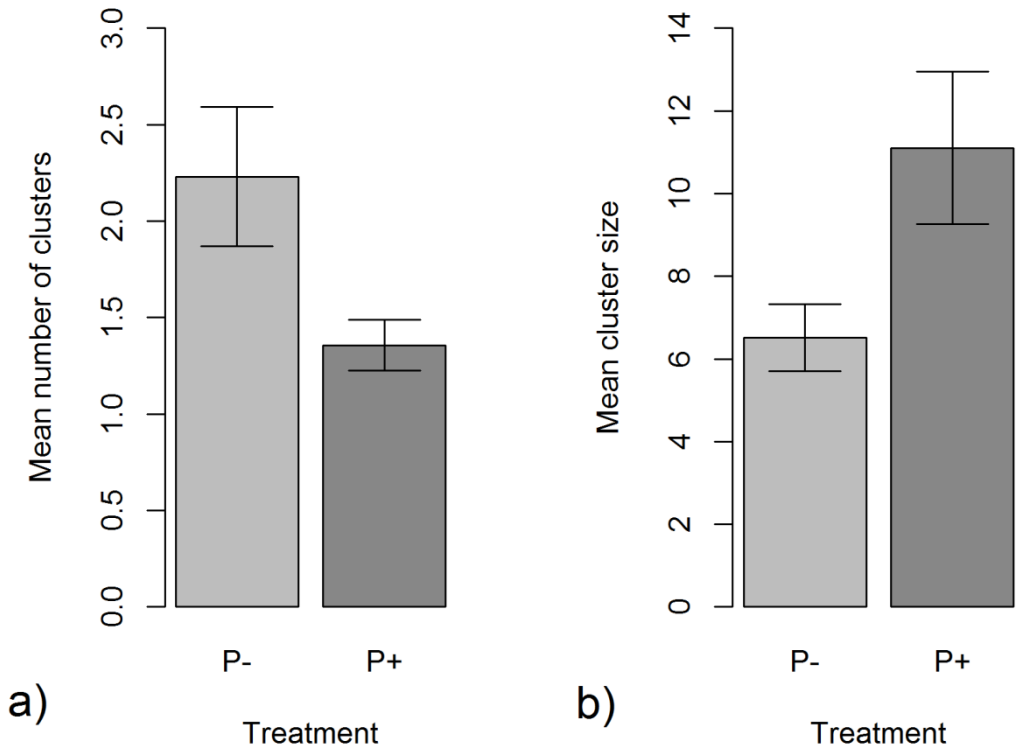

Figure 1. a) mean (± SE) number of clusters laid per female and b) mean size (± SE) of clusters laid per female under conditions of either no predation risk (P-) or simulated predation risk (P+).

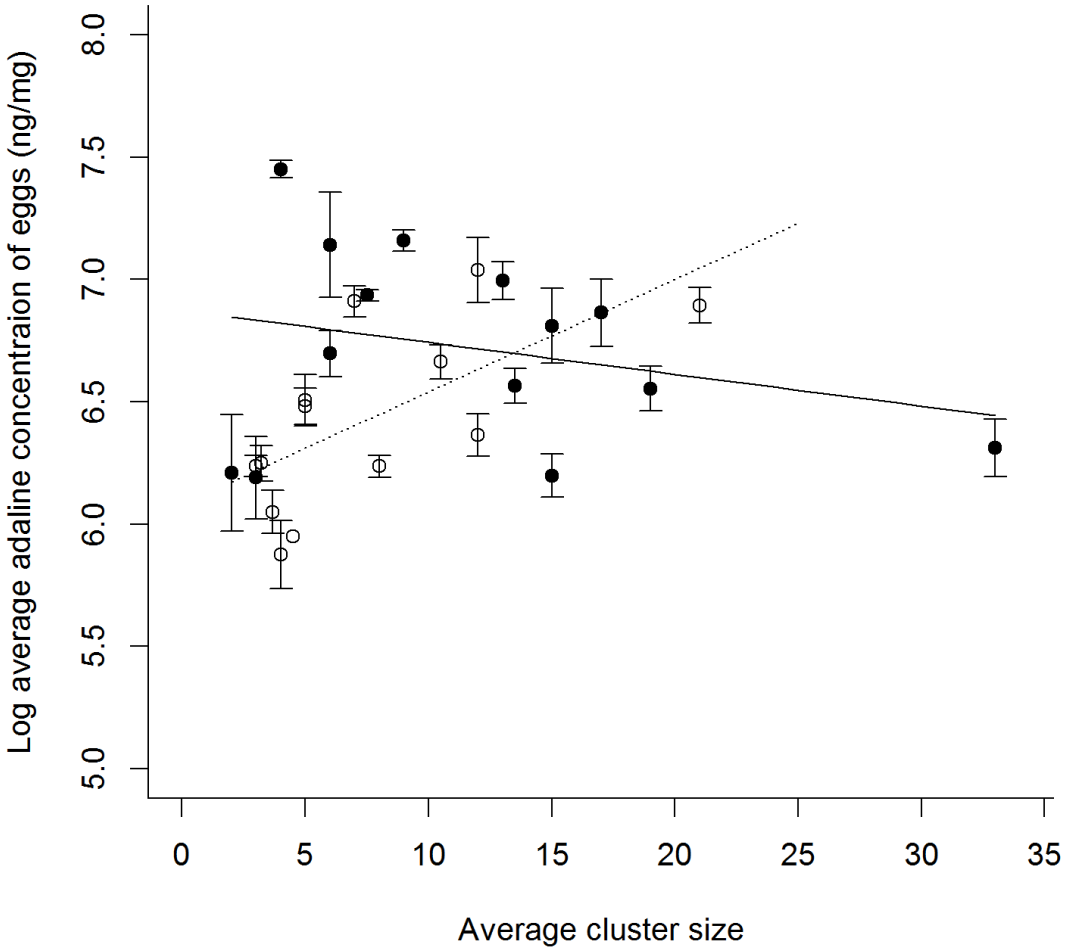

Figure 2. Mean egg adaline concentration (ng/mg, + SE) in relation to mean cluster size, per female under conditions of either no predation risk (○, ---) or simulated predation risk (●,—). Trend lines are back transformed predictions from glmm controlling for effects of female and cluster ID.

Discussion

Simulated predation risk did not affect either the number of eggs laid by females or the time at which they began to lay eggs. However, the way in which eggs were distributed amongst clusters did, with females laying fewer larger clusters under conditions of simulated predation risk than when predator cues were absent. The mean size of clusters laid by females was positively correlated with egg mass irrespective of treatment. Additionally, under conditions free from predation risk, there was a positive relationship between mean cluster size and egg toxin level whereas, in contrast, under conditions of simulated predation risk the slope of the relationship between mean cluster size and toxin level was negatively signed.

The positive relationship between mean cluster size and egg adaline levels under P- conditions indicates that, in a risk-free environment, cluster size could be a 'quantitatively honest' signal of egg toxin level, where, in relation to defence against predators, stronger or more conspicuous signals are associated with better defended individuals (Holen & Svennungsen 2012). Such signalling honesty is thought to be maintained by the differential costs and benefits of signalling (handicap principal; (Zahavi 1975; Holen & Svennungsen 2012) where either: stronger signallers suffer more attacks but lower mortality than weaker signallers due to predator rejection after handling prey ('go slow' mechanism; (Guilford 1994)); or physiological coupling between the signal and the defence selects for stronger signallers to suffer fewer attacks and lower mortality than weaker signallers (resource allocation model; (Blount *et al.* 2009)). In the case of cluster size either mechanism could be involved. The size of the cluster itself may send a stronger or more 'efficient' deterrent signal to predators, either chemically or visually, as demonstrated by the aggregation of aposematic individuals (Gamberale & Tullberg 1996). This in turn may cause

predators to be cautious and ‘go slow’ when attacking larger clusters, the eggs of which they are more likely to reject, thus increasing the survival of eggs in larger clusters. Alternatively, eggs are expensive to produce (Monaghan *et al.* 1998; Messina & Slade 1999) as are toxins (Holloway *et al.* 1991b), and so increasing cluster size would be likely to involve a concomitant decrease in egg toxin level due to the finite resources available (Blount *et al.* 2009). Models have demonstrated that such resource allocation trade-offs between signal and defence can lead to an evolutionary stable strategy where individuals allocate resources optimally between defence and signalling, resulting in a positive correlation between the two (Blount *et al.* 2009; Holen & Svennungsen 2012).

In contrast, the negatively signed slope under P+ conditions suggests that, in the presence of predator cues, signal honesty broke down and cluster size was no longer a reliable signal of egg toxin levels. We suggest three possible explanations for the negative relationship between mean cluster size and egg toxin level under conditions of simulated predation risk (P+). Firstly, it is possible that P+ females withheld investment in costly toxins (Holloway *et al.* 1991b) as an example of ‘selfish maternal effects’ (from now on SMEs) (Marshall & Uller 2007)[23]. Though increased *A. bipunctata* egg toxin levels have been linked to reduced consumption by predators (Cottrell 2007), *H. axyridis* larvae are voracious, have high tolerance of novel alkaloids (Rieder *et al.* 2008; Sloggett & Davis 2010) and show limited discrimination between eggs of varying toxicity (Sloggett *et al.* 2009). Consequently, modulation of toxin investment in eggs may not alter egg survival prospects in the face of this particular predator. It may therefore be more beneficial to withhold investment, in order to conserve resources for future reproductive events in a potentially less risky environment (Marshall & Uller 2007), a strategy also shown by

females in other taxa after mating with poor quality males (Cunningham & Russell 2000; Uller *et al.* 2005). If P+ females were withholding investment, however, a reduction in the mass and total number of eggs laid may also be expected, compared with P- females, but this was not found.

Secondly, as cluster size 'honestly' signalled egg defence under P- conditions it is possible that under P+ females laid larger clusters to increase perceived levels of egg defence and therefore reduce predation risk, in an act of intraspecific Batesian mimicry or automimicry (Brower *et al.* 1970). Theoretical and empirical work has demonstrated that low levels of such automimicry can persist in populations (Lev-Yadun 2003; Gamberale-Stille & Guilford 2004; Speed *et al.* 2006; Svennungsen & Holen 2007; Jones *et al.* 2013). 'Cheats' (aka automimics) benefit from assuming the signal of better defended conspecifics and, though they degrade the 'common good', non-cheating conspecifics are still more likely to survive predation attempts due to their higher levels of defence and therefore unpalatability (Skelhorn & Rowe 2007a). Speed and Franks (Speed & Franks 2014) have also recently argued that automimicry rather than reaching a stable equilibrium between cheats and non-cheats persists in populations as a result of antagonistic co-evolution, which leads to an evolutionary chase between individuals with poor levels of chemical defence and individuals with high levels of chemical defence. The result is a mixture of 'honest' and 'dishonest' signallers within the population, depending on the co-evolutionary cycle's progress.

Though frequently used to explain the diversity of defence and associated warning colouration seen in aposematic populations (Holen 2013), automimicry may also apply to other visual signals, such as cluster size. It is however, worth noting that ladybird eggs are aposematic (Winters *et al.* 2014b) and a component of any

deterrent signal given by larger cluster size may not merely be a property of the size of the cluster itself but also of its conspicuousness. Aggregation of aposematic individuals improves predator deterrence by increasing the efficiency of the aposematic signal (Gamberale & Tullberg 1996; Rowland *et al.* 2013). Conspicuousness as well as cluster size may, therefore, be an important component of signalling the toxin level of eggs in a cluster, an important consideration for future work.

What is not immediately obvious, is why laying larger clusters, as seen under P+ conditions, would be associated with a decrease in toxin level, i.e. why did females under P+ conditions cheat? One explanation is that a physiological trade-off between cluster size and egg toxin level became manifest in P+ females and not P- females, as the former laid significantly larger clusters than the latter. Alkaloid toxins are energetically expensive to produce (Holloway *et al.* 1991b; Blum & Hilker 2002), so there may have been a limit to the quantity of toxins females could produce per reproductive event. Therefore any increase in the number of eggs laid per discrete laying event, i.e. an increase in cluster size, may have reduced per egg toxin allocation. Examples of such physiological restriction in egg investment have been recorded previously, for example in lesser black-backed gull (*Larus fuscus*) eggs, where egg lipid content increased and yolk-to-albumen ratio decreased with increasing egg number (Nager *et al.* 2000; Verboven *et al.* 2010).

Thirdly, P+ females may have laid larger clusters for reasons other than automimicry of larger and more toxic clusters. Egg clustering by insects can decrease predation (Stamp 1980; Sillentullberg 1988) including predation of ladybird eggs by heterospecific larvae (Agarwala & Dixon 1993) and, in addition to stronger aposematic signals, the so called ‘avoidance’ and ‘dilution’ effects are thought to be

key to this reduction in predation (Turner & Pitcher 1986). The avoidance effect is a reduction in the likelihood of a predator encountering a group or cluster of prey than an equal number of individual solitary prey (Johannesen *et al.* 2014). Even if a predator then detects a prey aggregation it is also unlikely to be able to consume all of the prey, increasing the proportion of prey individuals that survive compared to an attack on fewer or lone prey, a.k.a. the 'dilution' effect (Bertram 1978; Turner & Pitcher 1986; Rodgers *et al.* 2010). Both effects can also counterbalance the effect of higher detection rates resulting from the aforementioned stronger deterrent signals produced by clusters (Riipi *et al.* 2001). Increasing egg cluster size under P+ conditions could therefore, have been an effective anti-predator strategy irrespective of changes in aposematic signal strength. Again the possible physiological cost of producing large clusters can be invoked here to help explain the concomitant reduction in egg toxin level with increasing cluster size under P+ conditions.

It is also worth noting that ladybirds can lay infertile eggs. This infertility can be caused by STIs, such as Wollbachia sp. (Werren *et al.* 1994; Hurst *et al.* 1999), or be the result of trophic egg laying on the part of the female. Trophic eggs are infertile eggs laid by mothers in order to provide extra resources for their offspring (Perry & Roitberg 2006). The production of these eggs is an adaptation to poor resource conditions, and accordingly female ladybirds increase the number produced when laying in areas with low food availability (Perry & Roitberg 2005b). As trophic egg production is strongly associated with low aphid numbers, variation in the number of infertile eggs between the two treatment levels would not be expected *a priori* as this experiment did not manipulate resource availability, providing adlib aphids prior to and during the experiment. However, it is interesting that intraguild predators such as *H.axyridis*, are not only a source of predation risk for offspring but also of competition

for resources. The aphid colony will be being exploited, possibly heavily, by those ladybird larvae already present, and the more immediate risk of predation for offspring, will be superseded by low resource availability when offspring hatch. Females may therefore respond to intraguild predator presence by increasing the number of trophic eggs laid, another possible explanation for the larger clusters laid in the P+ treatment. The adaptive nature of such a strategy is however questionable as predatory larvae may consume the extra eggs. Additionally though previous trophic manipulation studies recorded changes in the proportion of trophic eggs per cluster there was no change in cluster size itself (Perry & Roitberg 2005b)[106]. Therefore the evidence to support the occurrence of trophic egg laying in this experiment is weak, but cannot be ruled out as egg toxin analysis is destructive. Additional work could therefore be carried out to ascertain whether predation risk does affect trophic egg laying.

The lack of difference between the two treatment levels in both total egg number and latency to lay, contrasts with previous studies using *A. bipunctata*, where the presence of heterospecific predators or their tracks delayed the onset of laying (Magro *et al.* 2007) and egg number was reduced as a consequence (Kajita *et al.* 2006). This discrepancy may be because our experimental females were slightly older than in the previous studies (Frechette *et al.* 2004; Martini *et al.* 2013), though still at the age of peak fecundity (Lanzoni *et al.* 2004), and only mated 24 h prior to the experimental start point. As a result they were likely to have been time-limited rather than egg-limited (Rosenheim 1999) and therefore may have been less discriminatory than younger individuals about the environments in which they laid (Mangel 1989; Sevenster *et al.* 1998).

Conclusions

In conclusion our results are the first demonstration that maternal exposure to heterospecific predation risk can influence toxin investment into eggs. Females increased cluster size but not toxin investment in eggs in the face of offspring predation risk, and the concomitant decrease in egg toxin level can be explained either via: 1) a reduction in investment due to SMEs, or 2) physiological constraint, where increases in cluster size, due to either the benefits of a) 'cheating' or b) the avoidance and dilution effects, caused a decrease in toxin level. Further work should focus on disentangling these possible explanations via: maternal resource manipulation, to assess whether constraint or SMEs were responsible for the reduction in toxicity associated with increased cluster size under P+ conditions; by assessment of whether egg, and therefore cluster signal strength (either visual or chemical), are honest signals of toxicity and how this varies under different predation conditions; and by manipulation of the strength of the cluster signal (again either visual or chemical) in predation experiments using *H. axyridis*, to establish whether cluster size influences survival by increasing signal strength or by avoidance and dilution effects. Finally this is the first demonstration that maternal effects are involved in the reproductive response of a native species exposed to an invasive predator of their offspring and future work is required in order to explicitly test whether this response increases or decreases maternal fitness i.e. whether it is adaptive or a form of evolutionary trap (Schlaepfer *et al.* 2002).

CHAPTER 3

Offspring food availability and invasive egg predators interactively affect maternal investment in egg chemical defence.

Abstract

Invasive species commonly predate the offspring of native species. Females can alter offspring phenotype in response to predators in a way which reduces predation risk, an example of maternal effects. However, changes in offspring phenotype that occur in response to a novel invasive predator are unlikely to be independent of other factors that drive maternal effects e.g. offspring food availability. Here we measured alterations in maternal investment of the ladybird *Adalia bipunctata*, including egg alkaloid levels, in response to invasive harlequin ladybird larvae, which prey upon *A. bipunctata* eggs, and differing levels of food (aphid) abundance. There is positive covariance between predator presence and food availability in the wild and, contrary to predictions, the results indicate that the response of females to one environmental factor is constrained by the response to the other. *A. bipunctata* females laid eggs with a higher alkaloid content in the absence of aphids, but only when predator cues were also absent. A palatability test showed that conspecific larvae preferentially consumed eggs of a higher toxin level, suggesting that females may increase alkaloid level to increase sibling cannibalism in the absence of aphids, as cannibalism benefits maternal fitness in low resource environments. This

response was likely constrained when predator cues were also present due to the larger number of eggs laid in the presence of predator cues resulting in a trade-off between egg number and egg alkaloid level. Our results demonstrate that maternal effects can facilitate species' responses to an invasive offspring predator and highlight the importance of studying maternal effects in the context of the multifaceted environments in which they occur.

Introduction

Ecosystems across the globe are undergoing rapid anthropogenically driven change (Steffen *et al.* 2007), exposing species to novel biotic and abiotic pressures, for example invasive species (Mack *et al.* 2000). While we need to understand the threats that these changes pose, they provide us with a unique opportunity to study how organisms adapt to novel alterations in their environment (Visser 2008). Maternal effects enable the alteration of phenotypes across generations independent of genetic inheritance (Mousseau & Fox 1998; Wolf & Wade 2009), facilitating the rapid response of species to environmental change (Bernardo 1996a). It is becoming increasingly apparent that they play a key role in the response of species to anthropogenic change e.g. increases in temperature (Donelson *et al.* 2012) and oceanic CO_2 levels (Miller *et al.* 2012). Much less is known, however, about how maternal effects may mediate species responses to novel predators i.e. invasive species, a key component of global change (Mack *et al.* 2000).

Invasive species commonly prey upon the offspring of native species (e.g. Pell *et al.* 2008), and consequently can have profound effects on the abundance and persistence of native species (Paolucci, MacIsaac & Ricciardi 2013). Their impact depends heavily upon how well they can detect and then respond appropriately to invasive predators (i.e. prey naïveté; Carthey & Banks 2014). Naïveté is determined by the ecological novelty of the predator (Rehage, Dunlop & Loftus 2009), the diversity of native predators to which prey are exposed (Ferrari *et al.* 2007), and the adequacy of prey defence (Banks & Dickman 2007). The expectation is that due to a lack of shared evolutionary history, native prey are likely to have high naïveté to invasive predators (Cox & Lima 2006). However, where invasive predators produce similar visual and/or chemical cues to native predators, prey can adaptively adjust

their behaviour and morphology in response to invasive predator presence (Kovalenko *et al.* 2010). Mothers are already known to alter offspring phenotype, via maternal effects, in direct response to predation pressure and cues of native predator presence (Storm & Lima 2010); these changes in offspring phenotype maximising maternal reproductive success (e.g. Walsh *et al.* 2015). It is conceivable therefore that, under certain conditions, maternal effects may also play a role in the response of native species to invasive predators.

Any transgenerational response to an invasive predator is unlikely to occur independently of maternal responses to other environmental factors. Females reproduce in complex multidimensional environments where positively covarying factors can have opposing influences on offspring survival and phenotype, e.g. predation risk and favourable abiotic conditions (Touchon & Worley 2015). Consequently, focusing on maternal effects in the context of isolated single environmental variables shows only a small part of the picture (Lau *et al.* 2008). The effect of conflicting environmental factors on plastic phenotypes has been elegantly illustrated in studies of individual phenotypic plasticity, as opposed to the transgenerational phenotypic plasticity seen in maternal effects, in response to reliable environmental cues (Tollrian *et al.* 2015). Crossed gradients of environmental variables that favour opposing phenotypes typically result in individuals displaying a balance between the two phenotypic optima (Relyea 2004; Hoverman & Relyea 2016). It therefore seems reasonable to expect that changes in offspring phenotype, brought about by maternal effects, may themselves also be subject to antagonistic selection pressures on phenotypic optima. For example, increasing egg size may compensate for poor resource availability (Fox & Mousseau 1996), but may also make offspring more conspicuous or attractive to parasites (Otto

& Mackauer 1998). Empirical tests of how such dynamic trade-offs may determine maternal effects are however scarce, and even less is known about how they may be altered by anthropogenically driven environmental changes, e.g. the arrival of invasive species.

To fully understand female investment via maternal effects in the face of invasive offspring predators, reproductive decisions must therefore be studied in the context of the interactive and potentially antagonistic factors present in the mother's reproductive environment (Deas & Hunter 2013; Deas & Hunter 2014). The aposematic (chemically defended and warningly coloured) 2-spot ladybird, *Adalia bipunctata*, is a native UK ladybird and an ideal species with which to investigate such ideas. *A. bipunctata* are subject to multiple pressures during reproduction, including predation of their eggs by larvae of the recent UK invasive ladybird species *Harmonia axyridis, from hereafter harlequin* (Katsanis *et al.* 2013). Female *A. bipunctata* do not provide post-egg laying maternal care but lay clusters of eggs on plants supporting aphid colonies on which the offspring can feed post-hatching (Agarwala & Dixon 1993). They also provision eggs with defensive alkaloids (Paul, Pell & Blount 2015). However, areas of high food availability also have high levels of offspring predation risk (Smith & Gardiner 2013) and this positive covariance means that *A. bipunctata* mothers are faced with opposing pressures on both egg laying behaviour and offspring phenotype. Firstly, *A. bipunctata* females can avoid laying eggs in areas where there are offspring predators (Magro *et al.* 2007), but at the cost of food availability for emerging offspring. This may explain why, despite the deterrent effect of offspring predators, ladybirds will still lay eggs in the presence of predator cues when aphids are also present (Michaud & Jyoti 2007). Secondly, when laying in such resource-abundant environments in the presence of predator cues,

females benefit from minimising cannibalism (i.e. low egg alkaloid levels) and maximising predator deterrence (i.e. high egg alkaloid levels) (Kajita *et al.* 2010). There appears, therefore, to be a conflict between the optimal offspring phenotype when resources are abundant and the optimal offspring phenotype when egg predation risk is high. However, the degree to which each of these antagonistic pressures determines egg phenotype in *A. bipunctata*, via maternal effects, remains unknown.

Using a powerful factorial risk-by-resource design, we test whether *A. bipunctata* females alter oviposition behaviour and offspring phenotype in response to cues of invasive harlequin ladybird larvae, resource availability (aphid presence), and whether there is an interaction between the two factors. In a palatability experiment, we also assess whether *A. bipunctata* eggs with a higher alkaloid content are more vulnerable to cannibalism. This is crucial because, while it has been demonstrated that ladybird eggs with high alkaloid content are less palatable to predators (Kajita et al., 2010), prior work, although highly indicative, does not explicitly test whether increasing alkaloid content increases egg palatability for cannibals. We predicted that: 1) In the risk-by-resource experiment a) Females will be more likely to oviposit in the presence of predators when aphids are also present; b) Females will lay larger clusters of eggs when predators are present and clusters will be largest when predators are present and aphids absent; c) Egg alkaloid level will be greatest when perceived predation risk, and the selective benefit of cannibalism, are at their highest (i.e. resources are low) and smallest under the reverse conditions. 2) In the cannibalism experiment, eggs with higher alkaloid content will be preferentially cannibalised.

Methods

Insect Cultures

Stock cultures of *A. bipunctata* ladybirds, obtained from Gardening Naturally (Love Lane Industrial Estate, Cirencester, UK), and harlequin ladybirds, obtained from two well established wild UK invasive populations (collected at UK grid references SU6168 8950 and TL1253 1317) were maintained on an *ad libitum* diet of pea aphids (*Acyrthosiphon pisum*; reared on dwarf bean [*Vicia faba*] Sutton variety) at 18^{o}C in a 16L:8Dh light:dark regime.

Experimental protocols

Oviposition experiment

Experimental individuals were 1st generation virgin *A. bipunctata* adults of known age (19-29 days post eclosion) reared from stock culture individuals. Females were mated with a non-sib male (80 females and 80 males from five families) and 24h after pairing, females were weighed to the nearest 0.01mg (analytical balance GR-200 A&D® Gemini™) before being placed individually onto the focal plant in an experimental microcosm. Each enclosed experimental microcosm (38cmx23cmx17cm) contained two *V. faba* plants, the focal plant and the control plant, 12 $\pm$2.5cm in height and set 14cm apart from each other and 7cm from the tray edge. The focal plants were manipulated so that they varied in aphid abundance and, therefore, perceived predation risk.

There were four treatments:

(A+/P-) aphids and no perceived predation risk,

(A+/P+) aphids and perceived predation risk,

(A-/P-) no aphids and no perceived predation risk,

(A-/ P+). no aphids and perceived predation risk

The control plant was always clean, i.e. had no aphids and no perceived predation risk (A-/P-). A+ plants were infested with 60 pea aphids of mixed instars 4 days prior to the experimental start date. In P+ plants the perceived predation risk was achieved by attaching a filter paper (Fisherbrand QL100, 5cm diameter) on which the tracks of harlequin larvae had been deposited (from two unfed larvae that had been allowed to walk on the filter paper in a dish for 12h (Carter & Dixon 1982; Doumbia, Hemptinne & Dixon 1998; Magro *et al.* 2007). Papers with tracks and control papers (clean filter papers for use on P- plants), were each cut into 4 strips. These were attached by wrapping the paper either around stems or by folding either side of and flush to a leaf and stapling the paper to itself, such that the plants remained undamaged.

Females from different sib clusters were distributed evenly between the four treatments, so that morph and family ID were represented equally. Final sample sizes were as follows, A+/P-, N = 20; A+/P+, N = 18; A-/P-, N = 18; A-/ P+, N = 20, as four replicates failed due to escapees. Females were monitored for 9 hours: every 15min for the first 3h, every 30min for the subsequent 3h, and every hour for the final 3 h. Movement from the focal plant, the time of onset and location of oviposition, and the time that each of these behaviours was observed was recorded. Once they had

oviposited, females and eggs were removed, and all eggs were frozen at -80^{o}C prior to toxin analysis. All observations were made in a controlled temperature room (Adcocks Cereal Growth Chamber 2007, Adcocks, UK) at 21^{o}C in a 16L:8Dh light regime over 5 days, with four replicates of each treatment per day.

A. bipunctata eggs contain the toxic alkaloid adaline. To assay adaline each egg was weighed to the nearest 0.1µg using an electronic microbalance (Cahn C33; Scientific and Medical Products Ltd, Manchester, UK.) and homogenized for 30 seconds in 200µl of dichloromethane, using a handheld electronic pestle. Each sample was then centrifuged at 13RPM and 4^{o}C for 10 minutes. 100µl of solution was transferred into a screw-top auto-sampler vial. Samples (2µl) were analysed on a non-polar (HP-1, 50 m x 0.32 mm inner diameter x 0.5) Gas-Chromatograph (GC) (Agilent Technologies, UK) fitted with a cool-on-column injector, a deactivated HP-1 pre-column (1m x 0.53 mm inner diameter) and a flame ionisation detector (FID). The GC oven temperature was maintained at 30°C for 1 min after sample injection and then raised by 5°C min^{-1} to 150°C, then 10°C min^{-1} to 240°C. The carrier gas was hydrogen. Peak enhancement by co-injection with a pure adaline standard was used to confirm correct identification of the adaline peak. Absolute adaline concentration per egg (ng/mg) was quantified by transforming the peak area using a calibration curve created from an external standard of pure adaline in dichloromethane at the following concentrations; 100ng/µl, 50ng/µl, 10ng/µl, 5ng/µl, and 1ng/µl.

Cannibalism experiment

Recently eclosed 4th instar *A. bipunctata* larvae (n=161) were raised from 14 pairs of adults taken from the stock culture and fed on an *ad lib.* diet of pea aphids. Larvae were fed 24 h prior to the trial with (0.01g; ~ 40 aphids), to standardise hunger levels,

weighed to the nearest 0.1mg (Ohaus Explorer® e12140 balance, Ohaus Europe GmbH, Greifensee, Switzerland) and then placed individually into test arenas (55x10mm Petri dish) facing two eggs. The bidirectional choice test consisted of two conspecific eggs one with a high toxin content (H) and the other a low toxin content (L), set 20mm away from larvae. Eggs were set 20mm apart, the position of each egg was alternated between dishes to obviate side preference bias, and trials were carried out blindly with respect to the hypotheses under test and under uniform conditions (HQI lamps,700 µmols $m^{-2}s^{-1}$ at 18 $\pm$ 2 ^{o}C). Larvae were continuously monitored for 2 hours and the time at which they contacted an egg, the identity of the egg first contacted, the identity of the egg consumed, whether they consumed the entire egg and the duration of feeding, were recorded. Larvae were then immediately euthanized and stored at -80 ˚C. The body length of individuals was measured to the nearest 0.001mm using a Leica M165 C stereo microscope (Leica Micro Systems Ltd, Milton Keynes, UK), in order to enable the calculation of larvae body condition. This was calculated using the Scaled Mass Index ($\widehat{Mi} = Mi[\frac{Lo}{Li}]^{b_{SMA}}$), where Mi is the body mass and Li the length of an individual, Lo the arithmetic mean length of the study population, and bSMA is the scaling exponent estimated by the standardised major axis (SMA) regression of mass on length following Peig & Green (2009,2010). Fully accounting for the scaling relationship between mass and length. The *A. bipunctata* eggs used for the trial were collected from culture and frozen at -80˚C for 12 months. Quantification of egg adaline content is destructive, but within-female repeatability of egg adaline content and egg mass is high (Paul, Pell & Blount 2015). The values of egg adaline and egg mass from previously analysed eggs (Paul *et al*. unpublished data) were therefore used as proxy values for the females that laid them and used to select remaining unanalysed eggs for the cannibalism trials. To maximise egg toxin

difference between H and L eggs and minimise difference in egg mass, females were ranked based on these values and paired so that the values of adaline were more than four standard deviations (SD) apart and egg mass values were less than 1 SD apart.

Data analyses

Data were analysed using R version 3.2.2 (R Core Team, 2016). Data were examined for normality, homoscedasticity and outliers and where appropriate transformed to improve the normality of model residuals. The alpha level was set at 0.05 for all tests and stepwise backwards deletion was employed to reach the minimum adequate model (Crawley, 2013).

Oviposition experiment

All models contained perceived predation risk/absence of perceived risk (P+/P-), aphid presence/absence (A+/A-), and their interaction as fixed effects. Generalized linear models (GLM, package = MASS) with binomial errors and a log link function were used to assess whether these fixed effects and female age influenced: whether a female moved from the focal plant, whether females oviposited, and whether or not they oviposited on the focal plant. For those females that moved from the focal plant when aphids where absent and predator cues were present (A-/P+), this altered the laying environment of females in this treatment as 'larval tracks' are low volatility contact cues (Ruzicka 2002; Oliver *et al.* 2006). Analyses were therefore carried out with predation risk level altered to P- if females in the A-/P+ treatment moved from and oviposited off of the focal plant. The influence of the treatments and of female weight and age on the time of oviposition and total egg number was assessed using a GLM with negative binomial errors and a log link function (error structure used to

account for over-dispersion in each model). The effect of the treatments on the sqrt of both total egg alkaloid (adaline) level and egg alkaloid (adaline) concentration was fitted with the standard fixed effects mentioned above and GC run date, and female ID (multiple eggs per female were measured) as nested random effects using a general linear mixed effects model (LMER, package=lme4; Bates *et al.* 2015). Egg mass was included as a factor in the aforementioned model for total egg alkaloid level to assess the relationship between egg size and alkaloid content. Post-hoc comparisons of significant interaction terms were carried out using the 'multcomp' package in R (Hothorn, Bretz & Westfall 2008). Additionally, due to results from the main analysis on egg adaline, a further analysis was carried out to assess the relationship between egg adaline and total egg number in the absence of aphids (i.e. P-/A- and P+/A- treatments) using a LMER where GC run date, and female ID were nested random effects. The repeatability of alkaloid levels in eggs laid by each female was calculated in the 'rptR' package following (Nakagawa & Schielzeth, 2010).

Cannibalism experiment

A two-tailed binomial test was used to assess whether there was a difference in the consumption of high toxicity (H) and low toxicity (L) eggs in the bi-directional choice trials. A GLMM with a binomial error structure, logit link, and larval family ID as a random effect was fitted to assess whether larval body condition, the egg first contacted, and time of day (fixed effects) predicted whether an H or L toxicity egg was consumed in the trial. The effect of egg toxicity and Scaled Mass Index on consumption latency was analysed using GLMM with a Poisson error structure and log link function, where larval family ID and an observation level were included as

random effects. The observational random level effect was included as time data were over dispersed but were not zero-inflated (Harrison 2014).

Results

Oviposition experiment

Contrary to predictions there was no interactive effect of the aphid and predation risk treatments on oviposition behaviour (Table 1). A greater proportion of females oviposited when aphids were present than when they were absent (A+:95% and A-:68%, Table 1). Of females that oviposited (n=62), fewer laid on the focal plant when aphids were absent than when they were present (A+:97% and A-: 62%, Table 1). It also took females longer to initiate laying when aphids were absent than when they were present (A-: 349 $\pm$ 35 mins and A+: 223 $\pm$ 23 mins, mean $\pm$ SE; Table 1). Predation risk, but not aphid treatment, influenced the number of eggs laid. Females laid more eggs when exposed to simulated predation risk (P+) than when predation risk was not simulated (P-), and under both treatments female fecundity increased with female mass (Fig. 1, Table 1).

There was high within-female repeatability for total egg adaline (R=0.701, 95% CI = [0.583, 0.783], p<0.001), and egg adaline concentration (R=0.681, 95% CI = [0.561, 0.774], p<0.001), with confidence intervals well above zero. Both total egg adaline and egg adaline concentration were significantly higher when aphids were absent than when they were present but only when predators were absent (Figs 2 and 3, Table 1; P-A+ and P-A- pairwise with mean $\pm$ SE: 85 $\pm$ 22 (ng), Z= -2.53, p = 0.01 and 497 $\pm$ 167 (ng/mg), Z = -2.53, p = 0.01). Furthermore, there was no effect of egg mass on total egg adaline content (X^2_1 =1.33, p = 0.25), but for those females that laid eggs in the absence of aphids (i.e. P-/A- and P+/A- treatments) there was a

significant negative correlation between egg adaline concentration and the total number of eggs laid (Fig 4; $X^2_1 = 4.89$, p=0.027).

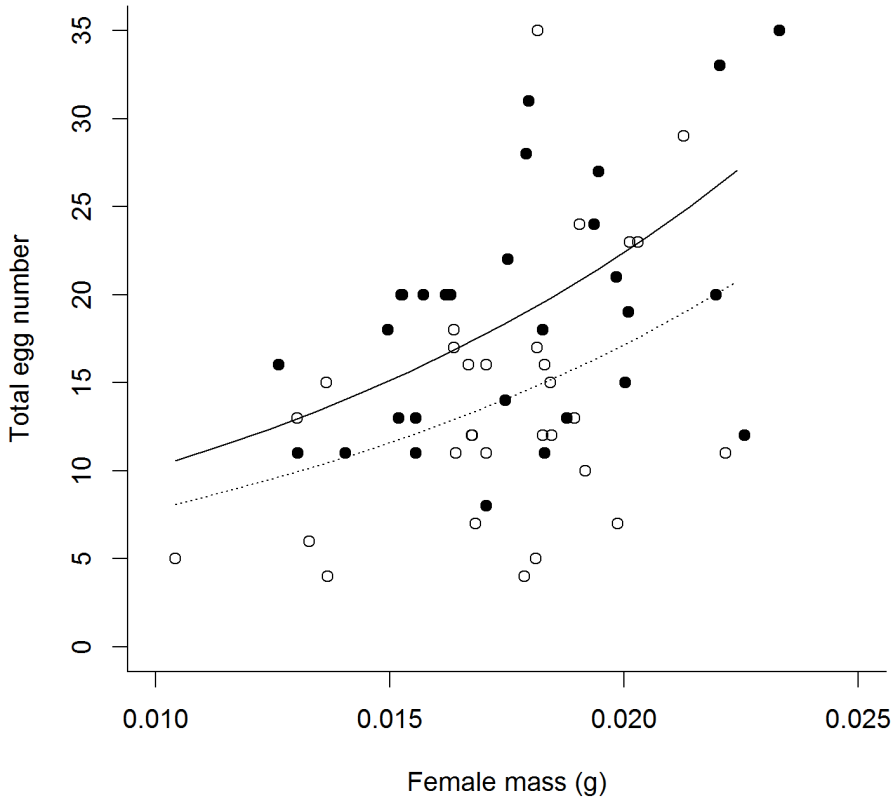

Figure 1. Total number of eggs laid by females under differing perceived predation risk environments (○ = no predation risk (P-), ● = predation risk (P+)), according female mass (mg) at the experimental start point. Plotted lines are estimates from GLM.

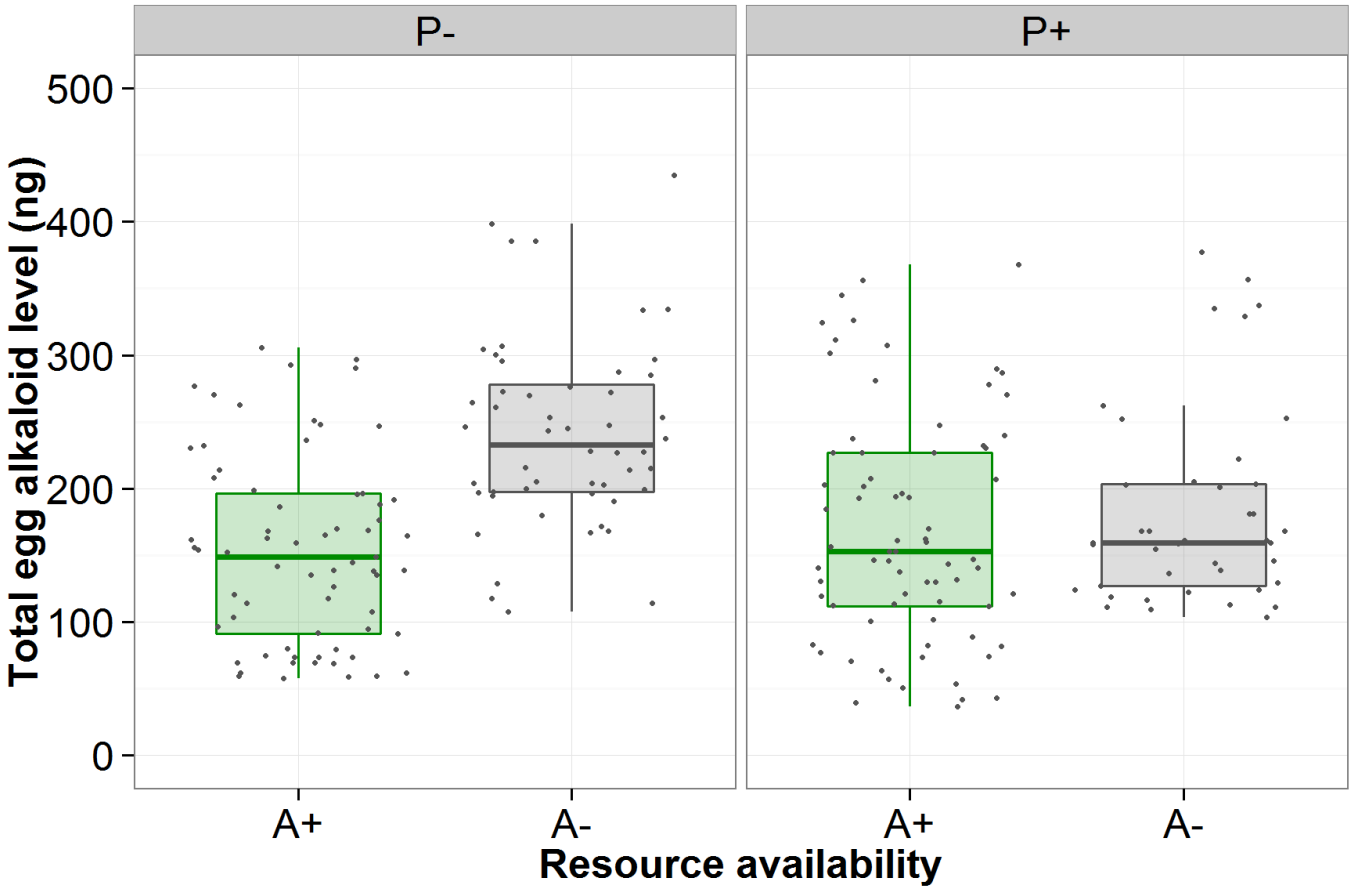

Figure 2. Effect of no perceived predation risk (P-) or perceived predation risk (P+) and aphid presence (green) or absence (grey) on levels of the alkaloid adaline in *A. bipunctata* eggs (ng).

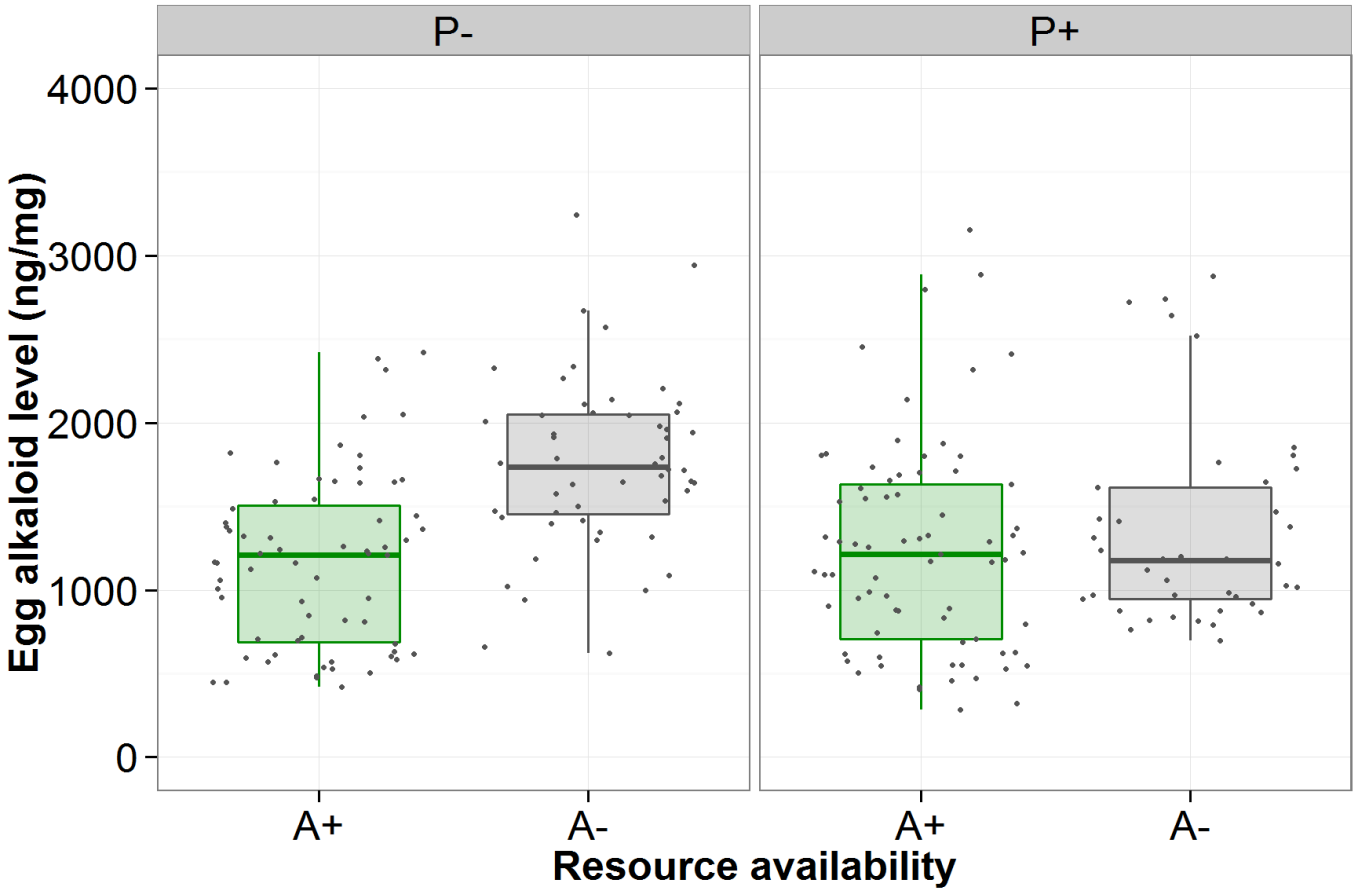

Figure 3. Effect of no perceived predation risk (P-) or perceived predation risk (P+) and aphid presence (green) or absence (grey) on the concentration of the alkaloid adaline in *A. bipunctata* eggs (ng/mg).

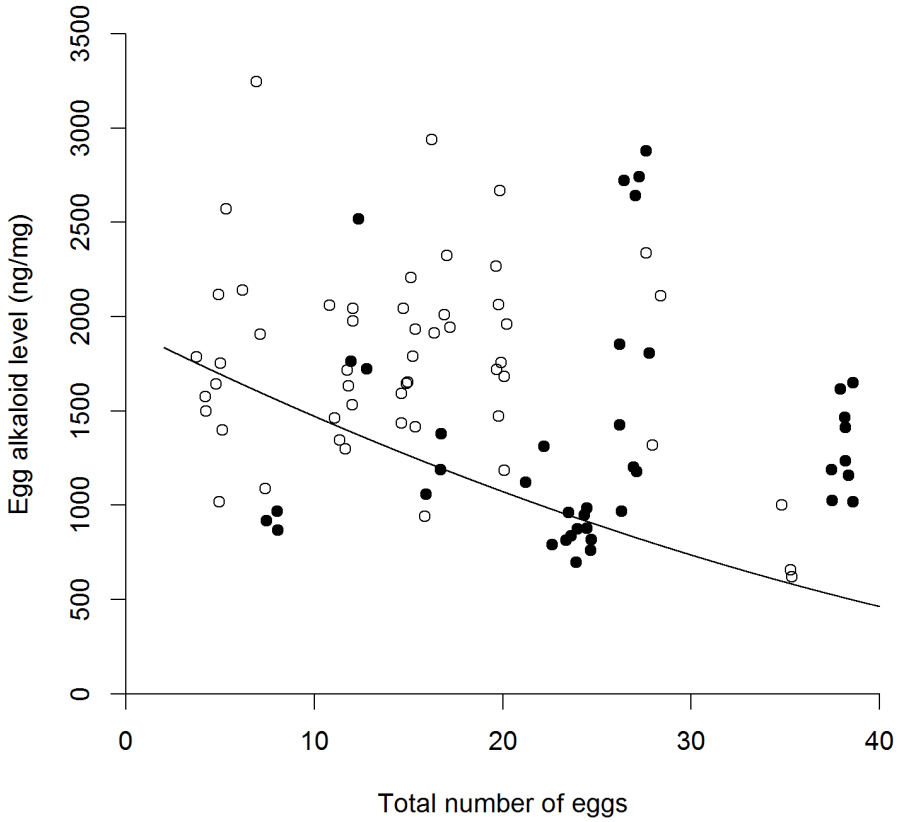

Figure 4. Relationship between the total number of eggs laid by *A. bipunctata* females and the concentration of the alkaloid adaline in those eggs (ng/mg) , in the absence of aphids (A-) and under different predation risk conditions (○ = no predation risk (P-), ● = predation risk (P+)).

Cannibalism experiment

132 out of the 161 larvae tested consumed an egg and, of the eggs eaten, significantly more contained high alkaloid levels (H) (p=0.018, probability of consuming H egg = 0.6, CI = 0.52-0.69), but larval body condition did not affect egg choice (X^2_1=0.23 P=0.63).

Factor	Movement from focal plant	Eggs laid	Egg laying on focal plant	Onset of egg laying	Total number of eggs	Alkaloid (adaline) ng/mg	Alkaloid (adaline) ng
Predation*Aphid	$X^2_{1,72} = 0.64$ p = 0.46 X	$X^2_{1,73} = 0.03$ p = 0.86 X	$X^2_{1,58} = 1.86$ p = 0.17 X	$F_{1,57} = 0.49$ p = 0.49 X	$X^2_{1,55} = 0.18$ p = 0.67 X	**$X^2_1 = 4.46$** **p = 0.03** ✓ *see pairwise comp*	**$X^2_1 = 5.57$** **p = 0.018** ✓*see pairwise comp*
Predation	$X^2_{1,74} = 0.29$ p = 0.590 X	$X^2_{1,74} = 0.20$ p = 0.65 X	$X^2_{1,60} = 0.15$ p = 0.70 X	$F_{1,58} = 1.00$ p = 0.32 X	**$X^2_{1,58} = 6.85$** **p <0.01** ✓ *More eggs laid*	NA	NA
Aphid	**$X^2_{1,75} = 14.02$** **p <0.001** ✓ *Females stay on focal plant*	**$X^2_{1,75} = 9.84$** **p = 0.002** ✓ *More females laid eggs*	**$X^2_{1,61} = 14.51$** **p <0.001** ✓ *More eggs laid on focal plant*	**$F_{1,57} = 6.15$** **p = 0.016** ✓ *Eggs laid rapidly*	$X^2_{1,57} = 0.22$ p = 0.64 X	NA	NA
Female weight	**NA**	**NA**	**NA**	**$F_{1,60} = -2.014$** **p = 0.048** ✓ *Egg laying earlier in larger females*	**$X^2_{1,58} = 16.16$** **p <0.001** ✓ *More eggs laid*	$X^2_1 = 3.20$ p = 0.07 X	**$X^2_1 = 4.21$** **p = 0.04** ✓
Female Age	$X^2_{1,73} = 0.81$ p = 0.42 X	$X^2_{1,73} = 0.23$ p = 0.63 X	$X^2_{1,59} = 2.60$ p = 0.11 X	$F_{1,59} = 1.49$ p = 0.23 X	$F_{1,56} = 0.19$ p = 0.67 X	$X^2_1 = 2.94$ p = 0.09 X	**$X^2_1 = 4.01$** **p = 0.04** ✓

Table 1. Summary of the effect of predation risk, aphid presence, their interaction and the effect of female age on the movement and oviposition behaviour of female ladybirds. Results are given as test statistics with associated df and p-value

Discussion

In egg laying species with no parental care, such as *A. bipunctata*, females employ two main strategies to maximise offspring survival in the face of predation risk; finely tuned oviposition site choice (Refsnider & Janzen 2010) and the alteration of egg phenotype (non-genetic maternal effects; Marshall & Uller 2007). These non-mutually exclusive strategies are used by species in response to the complex gradients of variability in the quality and availability of oviposition sites (Deas & Hunter 2013; Deas & Hunter 2014). Females of multiple species alter oviposition site in response to cues of offspring predation risk (Refsnider & Janzen 2010). This risk is rarely independent of other environmental factors that influence offspring survival, e.g. competition, which in turn can influence a female's oviposition response to predation (e.g. Binckley & Resetarits 2008). In female *A. bipunctata* ladybirds, however, predation risk and resource availability did not interact to affect female oviposition location or timing. The only detectable response to predation risk was that females laid more eggs. Females were more likely to lay eggs, and lay eggs quickly, in the presence of aphids, irrespective of predator cues, emphasising the importance of resource abundance to ovipositing ladybirds (Michaud & Jyoti 2007).

In contrast resource availability and predation risk interacted to affect egg phenotype, specifically egg alkaloid level. First and foremost the results of the *A. bipunctata* egg cannibalism experiment indicated that, as predicted, there is antagonism between the role of ladybird egg alkaloid level as a predator deterrent and cannibal attractant. Eggs with high alkaloid levels were preferred over eggs with lower alkaloid levels by conspecific larvae. This result is

consistent with the finding that, in addition to the general benefits of cannibalism (Ware, Yguel & Majerus 2009), cannibalistic larvae that consume high alkaloid eggs have greater alkaloid levels themselves (Kajita *et al.* 2010), thus promoting survival (Marples, Vanveelen & Brakefield 1994). The role of egg alkaloid level and toxicity in determining consumption by heterospecific ladybird larvae is well known (Sato & Dixon 2004; Kajita *et al.* 2010; Katsanis *et al.* 2013), but this is the first demonstration that cannibals (conspecifics) distinguish between, and preferentially consume, eggs with a high toxin level. The contribution of additional egg attributes, other than egg mass (see methods), to the preference shown by cannibalistic larvae, e.g. carotenoids (Winters *et al.* 2014), cannot be ruled out. However, the highly chemically motivated nature of larvae (Cottrell, 2007) and the positive fitness impacts of consuming high alkaloid content eggs (Kajita *et al.* 2010), strongly support the contention that: a) egg alkaloid levels play a role in the determination of egg consumption by cannibals, and b) there is therefore a conflict between the optimal egg alkaloid level when resources are abundant and the optimal egg alkaloid level when egg predation risk is high.

Under such conditions we predicted that egg alkaloid levels would be greatest when perceived predation risk and the selective benefit of cannibalism are at their highest (i.e. P+/A-) and smallest under the reverse conditions (i.e. P-/A+). Previous work on individual phenotypic plasticity has demonstrated such graded responses to antagonistic selection pressures on optimal phenotype, e.g. in the freshwater snail *Helisoma trivolvis* where different predators select for differing shell morphology (shell thickness vs width; Hoverman & Relyea 2016).

However, although there was an interactive effect of resource availability and cues of predation risk on egg alkaloid content, it was not in the direction predicted. Egg alkaloid level was greatest in the absence of aphids but, contrary to predictions, this was only the case when cues of predation risk were absent (as opposed to present) at the site of oviposition.

That egg alkaloid level was highest when aphids were absent, i.e. when resources were low and the fitness benefits of cannibalism were high, further supports the idea that egg alkaloids play a role in sibling cannibalism. Although consistently beneficial to the cannibalistic offspring, it does not benefit maternal fitness, or the fitness of the siblings that are eaten, for offspring to cannibalise when resources are abundant enough for fecundity to be maximised without it (Hamilton 1964; Pfennig 1997). This parent-offspring conflict has resulted in the evolution of mechanisms whereby mothers can manipulate levels of cannibalism in response to environmental conditions (Crespi 1992). For example, mothers can increase the number of available offspring to be cannibalised by laying trophic eggs (Perry & Roitberg 2005) or by increasing hatching asynchrony (Michaud & Grant 2004). Here, by increasing egg alkaloid content when aphids were absent, *A. bipunctata* mothers altered offspring phenotype in a way which would have potentially increased levels of sibling cannibalism under conditions where cannibalism was beneficial for maternal fitness. These results add to an emerging body of work examining other mechanisms by which levels of cannibalism are maternally fine-tuned (Wong, Lucas & Koelliker 2014), but are the first to indicate that an alteration in offspring 'quality' (alkaloid level) can be used by females to promote levels of

cannibalism, i.e. a ‘selfish’ maternal effect (Marshall & Uller 2007). It is also worth noting that both hatching asynchrony and the laying of trophic eggs occur in other ladybird species (Perry & Roitberg 2005). Neither have previously been detected in *A. bipunctata* and are not directly investigated here, as egg toxin analysis is destructive, but future studies should investigate whether they work in concert with egg toxin level to influence sibling cannibalism level.

Considering the benefits of offspring cannibalism to maternal fitness under low resources, it is not immediately clear why changes in an aspect of egg phenotype (egg alkaloid level) linked to increased levels of offspring cannibalism in *A. bipunctata* should only be observed in the absence of aphids when predators are also absent. The lack of increase in egg alkaloid level when aphids were absent but predator cues were present (i.e. P+/A-) may plausibly have resulted from the larger number of eggs laid in the presence of predator cues constraining any concomitant increase in female investment in egg alkaloid level (Smith & Fretwell 1974). Classic life-history theory predicts a context dependent trade-off between offspring number and levels of per-offspring maternal investment (e.g. size; Parker & Begon 1986; Bernardo 1996b), that has been empirically demonstrated. For example, the seed beetle *Strator limbatus* lays fewer but larger eggs when laying on tough seeds compared to pliable seeds; the larger eggs developing into larger larvae that are more likely to penetrate the thick seed coat (Fox, Thakar & Mousseau 1997). Here, for those eggs laid by *A. bipunctata* in the absence of aphids (A-) there was a negative correlation between the number of eggs laid and egg alkaloid level. This trade-off between egg number and egg alkaloid content, brought

about by the increased number of eggs laid in the presence of predator cues (P+), indicates that predation risk constrains response to the trophic environment and further emphasises the context dependent nature of maternal effects.

Alternatively, although it seems unlikely, we cannot rule out the possibility that a facultative reduction in female investment in environments of high offspring predation risk, may also have caused the observed difference in egg alkaloid level between the different predator treatments when aphids were absent. The total reproductive capacity of females is finite and therefore a reduction in offspring investment at risky or poor oviposition sites can enable them to increase their investment at more favourable sites, thus maximising female fitness (Rosenheim 1999). This does not necessarily involve the cessation of oviposition at risky sites, but a reduction, for example in offspring number (Guo *et al.* 2014), or in a costly aspect of offspring phenotype (Deas & Hunter 2014). However, if *A. bipunctata* females were employing this strategy in the face of predation risk a reduction in egg alkaloid levels between predator treatments when aphids were present as well as absent, and in other markers of investment, such as egg size or number, may reasonably have been expected, but this was not seen. In fact, there was actually an increase in the number of eggs laid when predators were present.

The response of *A. bipunctata* mothers to the risk posed by the presence of a novel offspring predator, was likely to have been enabled by the strong similarity between the chemical cues of larval harlequins and those of native predatory ladybird larvae (Magro *et al.* 2010). Similarity between familiar and

novel predators in terms of their ecology and behaviour is crucial in preventing the absolute naïveté of prey species to invasive predators, when there is no shared evolutionary history (Rehage, Dunlop & Loftus 2009). However, the naïveté of any native species to an invasive predator is comprised not only of their ability to respond to the presence of that predator, but the appropriateness of the response induced (Carthey & Banks 2014). Although increasing egg alkaloid levels benefits egg survival, through decreased palatability to predators, increasing egg number can also raise the total number of surviving offspring via the dilution effect, where the sheer number of prey prevents predators consuming them all (Turner & Pitcher 1986; Daly *et al.* 2012). However, native predatory larvae and harlequin larvae, though they may produce similar chemical cues, differ markedly in their tolerance to heterospecific egg alkaloids and in their voracity. Harlequin larvae, though certainly not unaffected by heterospecific egg alkaloids such as the adaline in *A. bipunctata* eggs, have a higher tolerance of alkaloids than native predatory ladybird larvae e.g. *Coccinella septempunctata* (Sato & Dixon 2004; Ware, Yguel & Majerus 2009; Katsanis *et al.* 2013). The higher palatability of *A. bipunctata* eggs to harlequin larvae (Agarwala & Dixon 1992) means that while consumption is likely restricted by the toxin burden accrued by larvae (Barnett *et al.* 2012), they may reasonably be expected to consume greater numbers of *A. bipunctata* eggs than native larval ladybird predators (Pell *et al.* 2008). Increasing egg number is therefore unlikely to be beneficial in the presence of harlequin larvae, although further tests of harlequin consumption rates compared to the increases in egg number seen here are needed to confirm this. Therefore, although *A. bipunctata*

can detect the presence of, and respond to novel invasive offspring predators, the response may not be adequate to prevent detrimental effects of harlequin larval predation on *A. bipunctata* reproduction. Data on the current status of UK *A. bipunctata* populations support this tentative conclusion. Indeed, *A. bipunctata* are in decline in the UK and populations are lowest in areas where the invasive harlequin has become established (Roy *et al.* 2012).

In conclusion, *A. bipunctata* can respond to an invasive offspring predator via maternal effects, though whether this response is adaptive is less certain. There is also an interactive effect of resource availability and invasive predator risk on maternal reproductive investment in *A. bipunctata*, but not in the direction predicted. The increased number of eggs laid under high predation risk constrains female responses to low resource availability, preventing an increase in toxin level. The results from the cannibalism test and the increase in egg alkaloid levels in the absence of aphids and predators, suggest that egg alkaloid levels are used by female *A. bipunctata* to manipulate offspring cannibalism levels in response to low resource availability. Overall these results emphasise the importance of studying the non-genetic transgenerational responses of species to anthropogenic change, such as those mediated by maternal effects, in the context of the other environmental factors that may be key in determining offspring phenotype.

CHAPTER 4

The hidden effects of melanism on brightly coloured offspring in an aposematic ladybird

Abstract

Contrary to theoretical predictions, species which colourfully advertise their chemical defence (aposematic species) have a variety of different colour morphs, which often vary in their degree of melanism. Despite their increased risk of predation, melanic morphs are prevalent throughout populations of aposematic species. Their persistent is thought to be due to other selective advantages, e.g. thermoregulatory benefits, however the role of juvenile phenotype in determining melanic morph abundance has been comparatively underexplored. This is surprising considering that firstly, selection acts at every stage of an organism's life cycle and secondly aposematic species commonly have complex lifecycles where the phenotype of early life stages can differ dramatically from that of the adults. We investigate differences in the phenotype of eggs laid by melanic and non melanic morphs of the aposematic ladybird *Adalia bipunctata.* We also concurrently assessed responses of females to high and low offspring predation risk from larvae of the invasive ladybird predator, *Harmonia axyridis*. We provide the first demonstration that the level of offspring chemical defence and associated warning coloration differs between different morphs of the same aposematic species. Both morphs lay yellow-orange

chemically defended eggs, i.e. their eggs have a similar aposematic phenotype, but the eggs of melanic mothers had consistently lower toxin content, luminance, and saturation. There was no increased egg toxin level in response to an increased risk of egg predation by *H. axyridis* larvae across all females. The weaker aposematic signal of eggs laid by melanic individuals may mean that, like the adults, they have greater susceptibility to predation from native predators. However, the higher tolerance of *H. axyridis* larvae to egg defence compounds may mean that differences in egg coloration and defence between the morphs have a comparatively smaller influence on predation by invasive *H. axyridis* larvae than native predators. Further tests of palatability and toxicity are needed to confirm whether asymmetries in wild predation risk exist between the eggs of the two morphs and whether this may change with *H. axyridis* invasion.

Introduction

Aposematism is a phenomenon whereby conspicuous signals are associated with a secondary defence in order to deter predation (Poulton, 1980). Classically this involves the use of striking coloration to advertise the presence of unpalatable, noxious, or toxic chemical defences by a species (Ruxton *et al.* 2004). Such defences and their associated visual signals are found in a number of taxa (Marples 1993; Brodie & Janzen 1995; Summers & Clough 2001; Cortesi & Cheney 2010; Stankowich *et al.* 2011) and in all cases operate through the promotion of both initial predator aversion and learnt avoidance (Lindström *et al.*, 1999; Rowland *et al.* 2013). Predator learning is maximised if one particular visual rule can be apped to multiple individuals (Lindstrom *et al.* 2001), and theory therefore predicts that individuals within, and even across

closely related aposematic species should have similar appearances and levels of conspicuousness (Müller, 1878). Contrary to this expectation, and despite some supporting empirical data (i.e. Müllerian mimicry (Rowe *et al.* 2004; Stuckert *et al.* 2014)), many aposematic species have a large number of different morphs (e.g. Bezzerides *et al.* 2007) and show considerable diversity across clades (e.g. Cortesi & Cheney 2010).

In insects, variation in aposematic signals can be split into two main types: variation in the chromatic component (e.g. red, orange, or yellow) and variation in the achromatic component (e.g. extent of melanisation). Both are known to influence predation rates (Nokelainen *et al.* 2012; Hegna *et al.* 2013), but it is variation in melanism that we will focus on here. Melanic signal components have previously been argued to be important for internal signal contrast; i.e. the degree to which aposematic patterns stand out (Guilford, 2000). Such internal contrast has, however, repeatedly been demonstrated to be less important in predator deterrence than the conspicuousness of an individual's colour against its background (Arenas et al., 2014; Aronsson & Gamberale-Stille 2008; Hegna *et al.* 2011). The extent of an individual's melanisation is also positively correlated with predation risk (Hegna *et al.* 2013), especially in species with highly melanised morphs (Arenas *et al.* 2015). Positive selection pressures must therefore exist if the persistence of melanic morphs within aposematic populations is to be explained (though see: Sinervo & Lively 1996; Gray & McKinnon 2007; Nokelainen *et al.* 2014).

There is, for example, a thermal benefit to melanism, with melanic morphs warming up more quickly at ambient temperatures than non melanic morphs

(DeJong *et al.* 1996), resulting in higher fitness in cold environments (Clusella Trullas *et al.* 2007; Lindstedt *et al.* 2009). Some aposematic species even increase their degree of melanism in response to sustained drops in temperature during development (Michie *et al.* 2011). Melanism has also been linked to increased immunocompetence, for example in the greater wax moth (*Galleria mellonella*) (Dubovskiy *et al.* 2013). These benefits may contribute to the asymmetrical mate preference recorded in species with melanic morphs or differing levels of melanism (Wang *et al.* 2009; Saino *et al.* 2013; Culumber *et al.* 2014; Mishra & Omkar 2014).

The role of juvenile mortality in the maintenance of multiple morphs has, however, not previously been investigated. This is surprising considering that early life stages are the most vulnerable to predation (Roff 1992) and moreover that predators of juvenile and adult stages commonly differ (e.g. Hodek *et al.* 2012) and therefore are likely to have very different sensory modalities (Stevens 2007). The selection pressures acting upon different morphs may consequently vary between juvenile and adult stages (Phillips & Shine 2006). Furthermore, the fitness of these early life stages is determined not only by the genetic contribution of parents but also via their non genetic contribution i.e. maternal effects (Wolf & Wade 2009). This mechanism of transgenerational inheritance enables females to invest in offspring in an environment specific way, which in turn maximises maternal fitness (Marshall & Uller 2007). For instance, mothers have been documented to increase levels of offspring defence and therefore survival in the face of increased offspring predation risk (Storm & Lima 2010) and parasitism (Deas & Hunter 2012).

Using two morphs of the aposematic two spot ladybird (*Adalia bipunctata*), melanic (*melanic*) and red (*typica*) forms, here we examine whether reproductive investment varies between morphs. Melanic adults have thermoregulatory advantages over non-melanics (DeJong *et al.* 1996), but experience higher predation risk (Arenas *et al.* 2015). Both morphs lay clusters of brightly coloured (yellow-orange) and chemically defended eggs (alkaloid adaline), but it is not known whether these eggs of the different adult morphs also differ in their toxicity or predator perceived colouration. The toxicity of eggs varies between females (Hemptinne *et al.* 2000a) and has been linked to predation risk, with more toxic eggs being less vulnerable to predation from heterospecific ladybird larvae (Kajita *et al.* 2010), key egg predators (Seagraves 2009). Likewise there is also within and between female variation in per egg investment in carotenoids, compounds with multiple functions that act as pigments and contribute to the yellow-orange colour of ladybird eggs (Goodwin, 1984). Ladybird eggs are therefore seen as aposematic, colourfully advertising their chemical defence (Poulton, 1890; Winters et al., 2014).

Ladybird eggs also signal their toxicity honesty, i.e. egg colouration and toxin level positively correlate (Winters et al., 2014). This signalling honesty is likely a result of enforcement mechanisms e.g. ‘go slow’ sampling by predators which punishes cheaters (Guilford 1994; Speed & Franks 2014). Such mechanisms prevent females from investing in a way where colour no longer becomes representative of egg toxicity, i.e. from producing dishonestly signalling offspring and consequently any variation in egg toxin levels and egg colouration are likely to be concurrent.There may however be greater conflict between red morph

females and her eggs in terms of carotenoid allocation, as the dietary origin of carotenoids limits their availability and red females have a higher demand for carotenoids (Blount et al., 2012). Red morph females may consequently produce less colourful eggs with lower toxin levels than melanic morph females.

We therefore predict that: 1) melanic morph females will lay more colourful eggs with a higher toxin level than red morph females 2) in both morphs egg colouration and toxicity will correlate positively (i.e. honest signalling) as in other ladybird species, 3) females will lay more colourful eggs with higher toxin levels under conditions of high offspring predation risk

Methods and materials

A stock culture of *A. bipunctata* (f. typica and melanic (f.quadrimaculata and f.sexpustulata), obtained from Syngenta Bioline (Little Clacton, Essex CO16 9QG), was maintained in a cage on an *ad lib.* diet of pea aphids (*Acyrthosiphon pisum* reared on *Vicia faba*) at 20^{o}C with a 16L:8D h photoperiod. Experimental *A. bipunctata* individuals were 1st generation virgin adults of known age (15-25 days post eclosion) reared from individuals obtained from the stock culture: 102 females and 102 males reared from 30 different adult pairs (n=102; P+P+ =22, P+P- = 22, P-P+ = 32, P-P- = 26).Ladybirds show last sperm precedence and so each female was mated with a non-sibling male of the same morph. Morph type in *A.bipunctata* is genetically determined (Palmer, 1911) and both melanic forms are dominant to the red (typica) form (Majerus, 1994). After 24h females were removed and placed individually into an experimental Petri dish that differed in simulated predation risk (see below) and provided with *A. pisum ad*

lib. Females from different sibling clusters were distributed evenly between the treatment levels, so that family ID and mate ID were represented equally in all four treatments.

The treatment levels consisted of a mixture of high (P+) and low (P-) predation risk distributed across the 4 days of the experiment. Females were exposed to one predation risk condition for 2 days and then either the same or the alternative condition for the subsequent 2 days, giving the following four treatment levels 1) P+P+, 2) P+P-, 3) P-P+, 4) P-P-. To create an environment that conferred a simulated risk of predation (P+), 4th instar *H. axyridis* larvae were placed, without food, into individual sterile Petri dishes (9 cm diam.), each containing a semicircle of corrugated filter paper (9 cm diam.) and left for 24 h (Doumbia *et al.* 1998; Magro *et al.* 2007). Larval tracks consist of a mixture of large alkanes that leave a persistent cue, due to their slow oxidation rates, with females responding to tracks left at room temperature, that are up to a month in age (Hemptinne *et al.* 2001; Ruzicka 2002; Oliver *et al.* 2006; Ruzicka 2006). Despite this, in order to maximise signal longevity, after filter paper was removed it was stored under nitrogen in sealed in 50 ml tubes, at -80^{O}C. A control environment of no simulated predation risk (P-) consisted of a sterile Petri dish (9 cm diam.) and a clean semicircle of corrugated filter paper that had not been in contact with *H. axyridis* larvae.

Mated *A. bipunctata* females were placed individually into a P+ or P- Petri dish, depending on treatment level, for 2 days (48 h), after which point they were moved into a new petri dish with either high (P+) or low (P-) predation risk for a further 2 days (48hrs). On each of the four days (24hrs) of the experiment the

number of eggs, the number of clusters and singly laid eggs, and the size of clusters was recorded at 1, 3, 6, 9 and 24 h intervals. A cluster was classified as a group of two or more eggs, with each egg being in physical contact with at least one other egg in that cluster. All eggs recorded at each time point were then removed to prevent cannibalism and transferred to a 5^{O}C fridge for approx. ~24h prior to photography. After each egg was photographed it was stored at -80^{o}C prior to toxin analysis (see below for colour and toxin analysis method details). After the full four days of the experiment had elapsed, females were removed and stored at -80^{o}C prior to toxin analysis. All experiments were carried out in an incubator (Percival® model I-41LL, 505 Research Drive, Perry, IA 50220 USA) at 18^{o}C and a 16L:8D h photoperiod.

Quantifying egg colour

Individual eggs were photographed using a Nikon D7000 digital camera which had undergone a quartz conversion to provide ultraviolet (UV) light sensitivity (Advanced Camera Services, Norfolk, UK), fitted with a Nikon 105-mm Nikkor lens. For photographs in the visible spectrum the camera was fitted with an ultraviolet (UV) and infrared (IR) blocking filter (Baader Planetarium, Mammendorf, Germany UV/IR Cut filter; transmitting between 400 and 700 nm). For photographs in the UV part of the spectrum the camera was fitted with a UV pass IR blocking filter (Baader U filter; transmitting between 300 and 400 nm). A Spectralon™ 40% diffuse grey reflectance standard (Labsphere, Congleton, UK) was also photographed under an identical set-up and camera settings, prior to the capture of individual eggs images, as the small size of eggs precluded the inclusion of a standard within the egg images (Stevens et al. 2007). All

photographs were taken in a dark room using standardized lighting provided by a UV daylight lamp (Iwasaki eyeColor arc lamp (6500k), with UV coating removed) with eggs placed on a sheet of black ethylene-vinyl acetate (EVA), used for its low (<5%) UV reflectance.

Each image was linearized with respect to light intensity and equalized with respect to the grey standard (Stevens *et al.* 2007) using the programme ImageJ 1.47t and the Multispectral Image Calibration and Analysis Toolbox plugin (Troscianko & Stevens, 2015). The entirety of each egg was selected for analysis, using a specialised plugin, which also calculated egg volume (Troscianko 2014). It is important when investigating any changes in anti-predator coloration, to do so in the context of predator vision (Stevens *et al.* 2007; Arenas *et al.* 2015). As previously discussed, although ladybird eggs are frequently eaten by other invertebrates (Smith & Gardiner 2013b), the predator that this study is concerned with is a ladybird larvae. Consequently, linearized images were mapped to the predicted responses of the ladybird visual system, using the spectral sensitivity of the ladybird *Coccinella septempunctata* (Lin et al. 1992) using the approach developed in Troscianko & Stevens (2015). This mapping technique is highly accurate compared to modelling photon catch data with reflectance spectra (Stevens and Cuthill 2006; Stevens et al. 2014; Troscianko & Stevens 2015). Ladybirds have three classes of retinular cells sensitive to mediumwave (MW), shortwave (SW) and ultraviolet (UV) light (Lin *et al.* 1992). The presence of these three receptors mean that ladybirds are potentially trichromatic and possess colour vision, and experimental work has demonstrated that many ladybird species can discriminate between different

colours (Mondor & Warren 2000; Adedipe & Park 2010; Kemp & Cottrell 2015; Wang et al. 2015), though standardised colour discrimination tests are needed for robust confirmation. Moreover, this spectral sensitivity represents adult not larval ladybird sensitivities and ladybird larvae are the predators used in this experiment. The vision of coleopteran larvae in some species is known to be less developed than adult vision (Buschbeck 2014), though the larvae of those species that are both predatory and live in well lit environments still have relatively sophisticated visual systems (Paulus 1986; Mandapaka *et al.* 2006). Fourth instar ladybird larvae, for example, have been demonstrated to show similar foraging behaviour to adults in response to variation in light level and colour cues (Khalil *et al.* 1985; Harmon *et al.* 1998). Using adult ladybird cone catch values can therefore be used as a guide to indicate possible changes in egg colour that may be detectable by predatory 4th instar larvae.

As stated above, ladybird vision is potentially trichromatic, containing three classes of retinular cells sensitive to mediumwave (MW), shortwave (SW) and ultraviolet (UV) light (Lin *et al.* 1992). The receptor type used for luminance vision varies considerably between different insects, but is generally the most abundant of the retinular cell classes (Osorio & Vorobyev, 2005). In ladybirds this is the MW channel (~520nm), with six MW receptors to each one of the other two receptor types per adult ommatidium (Lin *et al.* 1992), and as such this receptor was used for luminance calculations for data extracted using ladybird vision. Luminance is here used to refer to a visual system dependent measure of achromatic variation, or perceived lightness (Osorio & Vorobyev 2005; Stevens 2011). Prior to calculations of saturation, single cone catch

values (for ladybird vision) were converted into proportions to remove absolute variation in brightness (Endler & Mielke 2005). The proportional cone catch values were then converted into two colour space coordinates (X, Y), giving each individual a location of colour in two dimensional colour space (Kelber *et al.* 2003; Endler & Mielke 2005). Saturation was calculated as the shortest Euclidian distance from the achromatic origin, with saturation being greater the further a point is from the origin. Unlike adults, eggs laid by different morphs are all brightly coloured in the lw end of the visible spectrum (~570-750nm) (Figure 1). As in previous studies (Winters et al., 2014) our a priori expectation was that there would be no difference in the *type* of pigment in eggs, and therefore the type of colour of eggs (i.e. hue), either between treatments or between female morphs, but that there would be differences in pigment quantity and therefore in luminance and saturation. Due to this and the strong correlation of the calculated values of hue with both luminance and saturation (Appendix III.), hue was excluded from the analysis.

Figure 1. Cluster of *A.bipunctata* eggs, laid by a melanic female

Quantifying levels of adaline

The defensive alkaloid in the eggs and adults of *A.bipunctata* is adaline. One to five eggs were randomly selected from the last cluster laid by a subset of females, on days 2 and 4 of the experiment, at which point females had been exposed to experimental treatments for a maximum of 48 hours (n=60: P+P+ =18, P+P- = 11, P-P+ = 15, P-P- = 16). Each egg was weighed to the nearest 0.1µg using an XP6U Ultra-microbalance (Mettler-Toledo) and homogenized using a hand held pestle (Fisherbrand™ Pellet Pestle™ Cordless Motor) for 30 s in 200µl chloroform with an internal standard of 1ng/µl E,Z-4,7 tridecadienyl acetate (Pherobank, 6700 AH Wageningen). Samples were then centrifuged at 17.7 x g for 3 min, and an aliquot (100µl) transferred into an autosampler vial. Similarly, the elytra were removed from each female, and the body was weighed to the nearest 0.01mg using an analytical balance (GR-200 A&D® Gemini™) before being homogenised for 60 seconds in 500µl chloroform with an internal standard of 1ng/ µl E,Z-4,7 tridecadienyl acetate. After homogenization a second 500µl of solvent solution was added. Each sample was then centrifuged at 17.7g and 13.3rpm for 3 minutes. 10µl of extract solution and 90µl of solvent solution was then transferred into an autosampler vial. Samples (2µl) were injected into an Agilent 7890A GC coupled with a 5975B MS fitted with an HP5-ms column (30mx0.25mmx0.25µm film thickness). The injection was in pulsed splitless mode, and the inlet temperature was 250^{o}C. The carrier gas was helium with a flow rate of 1.3 mL/min. The GC temperature programme was 50^{o}C at injection increasing to 140^{o}C at 20^{o}C/min, then from 140^{o}C to 280^{o}C at 5^{o}C/min. Mass spectra operated in SIM mode,

scanning for ions m/z (166.2 for Adaline) and (79. 1 for standard). Adaline (ng/mg body tissue) was quantified relative to the internal standard.

Statistical analysis

All analyses were carried out using R version 3.2.2 (R Development core team, 2015). Data were examined for normality, homoscedasticity and outliers. The alpha level was set at 0.05 for all tests and stepwise backwards deletion was employed to reach the minimum adequate model (Crawley, 2013). Analyses were carried out using general linear (package = MASS) and generalized linear mixed effects models (package=lme4 (Bates et al., 2004); see below for specific families) with post-hoc Tukey's tests (package=multcomp (Horthorn et al., 2008)) reported following (Cichini *et al.* 2011).

1) *Egg metrics, colour and toxin level*

There was statistically significant repeatability of egg adaline concentration and both measures of egg colour within females (Egg adaline: R = 0.750, SE = 0.057, CI = 0.609, 0.832, p = 0.001; Egg mass: R = 0.757, SE = 0.03,CI = [0.693, 0.809], p = 0.001; Luminance: R = 0.65, SE = 0.041, CI = [0.562, 0.723], p = 0.001; Saturation: R = 0.552, SE = 0.046, CI = [0.465, 0.642], p = 0.001), calculated in the 'rptR' package following (Nakagawa & Schielzeth 2010; Nakagawa & Schielzeth 2013). Results supported the use of a subsample of eggs as representative of the adaline levels and colour of eggs laid per female. A negative binomial glmm was fitted to the concentration of adaline in eggs (ng/mg), with treatment, day, treatment*day, female morph and female weight as fixed effects and female ID as a random effect. The effect of treatment, day,

treatment*day, female morph and female weight (fixed effects) and female ID (random effect) on luminance and saturation was assessed using lmm.

2) Female laying behaviour

There was no difference between the treatments or between the morphs in whether or not a female cannibalised her eggs (Treatment: Chi-Sq, X^2_1=2.01, df = 3, p = 0.57, Morph: Chi-Sq, X^2_1=0.06, df = 1, p = 0.80). However, though the presence or absence of cannibalism is easily assessed the specific number of eggs cannibalised could not be quantified, and therefore only females that did not cannibalise their eggs were included in the following analyses of female laying behaviour (n(Fem)=102). Additionally it is worth noting here that no eggs from partially cannibalized clusters were included in the egg toxin and colour analyses.

A binomial glmm was used to identify differences in both the number of females laying eggs and the number of females that laid single eggs, with treatment, day, treatment*day, female morph and female weight as fixed effects and female ID as a random effect. For those females that laid eggs, the total number of eggs and the number of single eggs laid were each fitted to negative binomial mixed effects models (package=lme4, function=glmer.nb), with treatment, day, treatment*day, female morph and female weight as fixed effects and female ID as a random effect. A generalized mixed effects model (package=lme4, function=glmer, family=poisson), with treatment, day, treatment*day, female morph and female weight as fixed effects and female ID as a random effect was used to assess changes in the maximum size of clusters laid by females.

Female weight, used as a key fixed effect in these models may also have varied between morphs, consequently an ANOVA was used to describe the relationship between the two.

Results

Egg toxin level and colour

Egg adaline levels increased from days 2 to 4, i.e. across the experimental period, however the degree of change between the two days was treatment dependent (Figure 2; Table 1). There was no significant increase between the days under P+P+ treatment, but under all other treatments the increase was significant, with the greatest effect seen in the P+P- treatment (Table 2). This increase in toxin level also varied between the morphs, with eggs laid by melanic females showing a greater increase in toxin level between the two time points while overall still having a lower toxin level than those eggs laid by typica females (Figure 2; Table 2).

Of the two egg colour metrics, treatment only significantly affected luminance. This was driven by the greater luminance of eggs laid under P+P+ than in P-P- treatments (Figure 3; Coefficient=1042.60, Standard Error= 353.10, z value = 2.95, p= 0.017), with no other treatment levels differing significantly in egg luminance values. Egg luminance also decreased between days 2 and 4 , i.e. across the experimental period, and was higher in eggs laid by typica than melanic adults (Figure 3; Table 1). There was an interactive effect of female morph and day on saturation (Table 1), driven by the difference between the saturation of eggs laid by melanic females during each time period and by the

different morphs at day 4 (Figure 5; Table 2). Saturation correlated positively with egg toxin level (adaline ng/mg), but there was no significant relationship between luminance and egg toxin level (Table 1). Additionally egg mass and size increased over the experimental period (Table 3) and typica females had greater mass than melanic females (ANOVA, $F_{1,100}$=23.25, p<0.001).

Table 1. Changes in egg toxin (adaline) level and colour metrics with treatment, experimental run day, female morph and female weight (egg toxin level also included in all colour metric analyses).

Factor	Egg adaline concentration (ng/mg)		Luminance		Saturation	
	X_1^2	p	X_1^2	p	X_1^2	p
Treatment *Day	**9.430**	**0.024**	4.825	0.185	1.017	0.797
Treatment	**/**	**/**	**9.020**	**0.029**	2.609	0.456
Day	**/**	**/**	**25.069**	**0.000**	**/**	**/**
Female Morph * Day	**5.799**	**0.016**	1.975	0.160	**7.550**	**0.006**
Female Morph	**/**	**/**	**10.485**	**0.001**	**/**	**/**
Fem Weight	0.074	0.785	**N/A**	**N/A**	**N/A**	**N/A**
ng_mg	**N/A**	**N/A**	1.497	0.221	**5.927**	**0.015**

Table 2. Results of post-hoc Tukey tests, on interactive effect of treatment and experimental run day on egg toxin level (ng/mg).

Treatment	Day	Treatment	Day	Coefficient	Standard error	z value	p
P+P+	2	P+P+	4	0.0269	0.0440	0.613	0.998
P+P-	**2**	**P+P-**	**4**	**0.1923**	**0.0503**	**3.824**	**0.002**
P-P+	2	P-P+	4	0.1125	0.0449	2.504	0.139
P-P-	**2**	**P-P-**	**4**	**0.1463**	**0.0447**	**3.275**	**0.015**

Table 3. Change in egg mass (mg) and egg volume (mm^3) with treatment, experimental run day, female morph and weight and the other egg metric (i.e. egg volume when egg mass was the response variable).

Factor	Egg mass (mg)		Egg volume (mm^3)	
	X_1^2	p	X_1^2	p
Treatment *Day	7.183	0.066	2.135	0.545
Treatment	4.866	0.182	3.387	0.336
Day	**7.578**	**0.006**	**3.616**	**0.057**
Female Morph * Day	0.335	0.563	0.176	0.675
Female Morph	0.059	0.808	1.302	0.254
Fem Weight	0.753	0.386	0.109	0.741
Egg mass (mg)/Egg volume (mm^3)	**83.237**	**<0.01**	**88.927**	**<0.01**

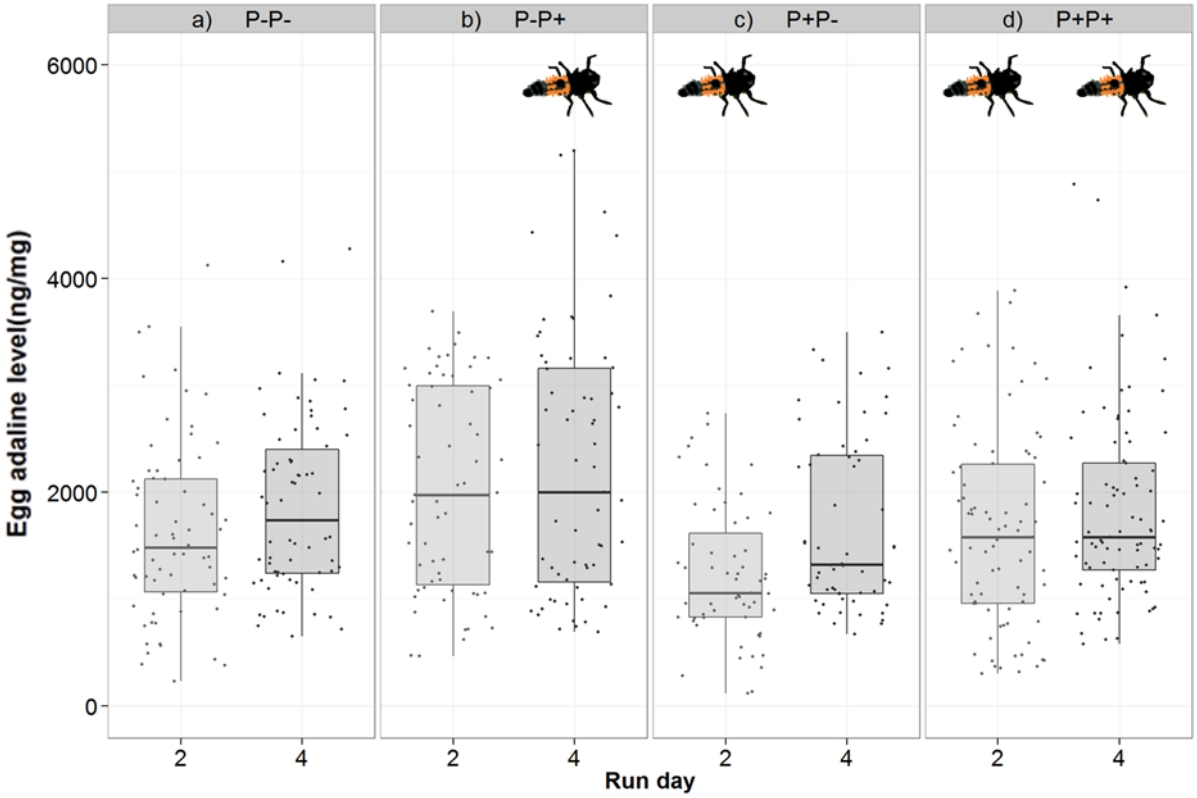

Figure 2. Change in egg toxin (adaline) level across the two halves of the experiment in each of the four treatments a) P-P, b) P-P+, c) P+P- and d) P+P+. Images of larvae denote periods in treatments where females were exposed to tracks of larval egg predator.

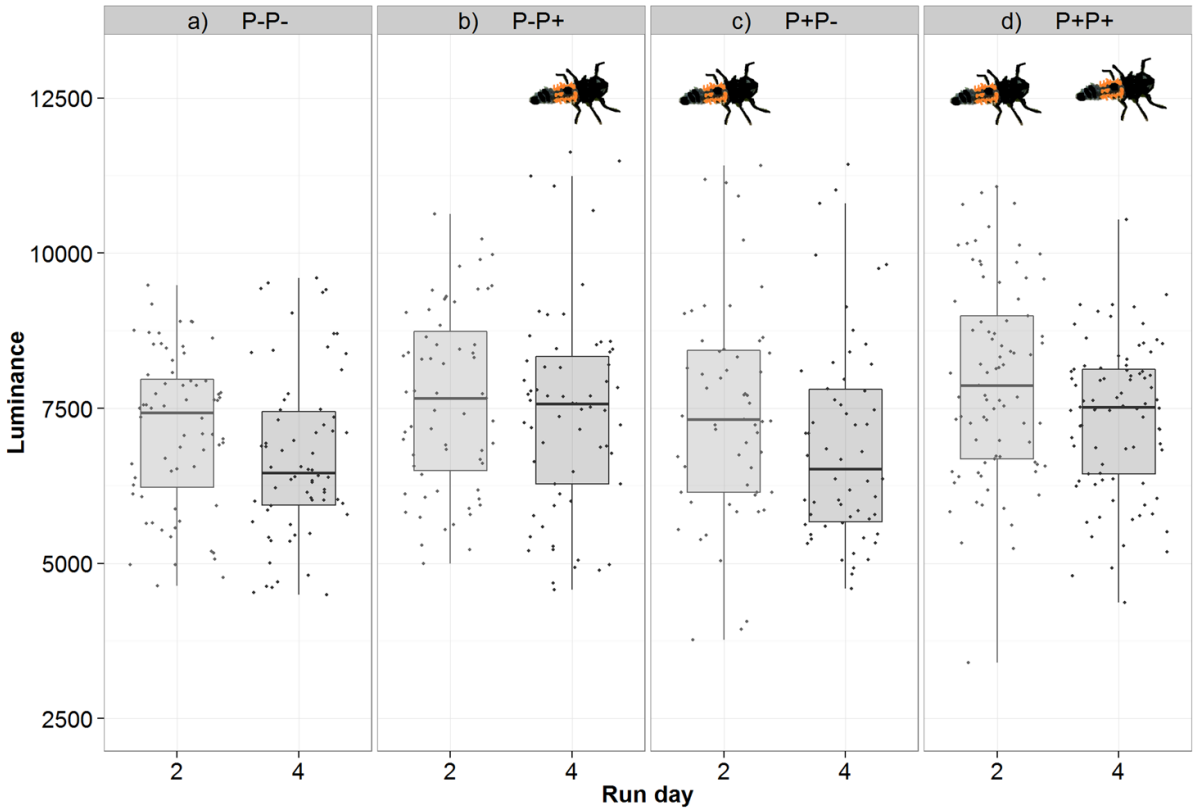

Figure 3. Change in egg luminance across the two halves of the experiment in each of the four treatments a) P-P, b) P-P+, c) P+P- and d) P+P+. Images of larvae denote periods in treatments where females were exposed to tracks of larval egg predators.

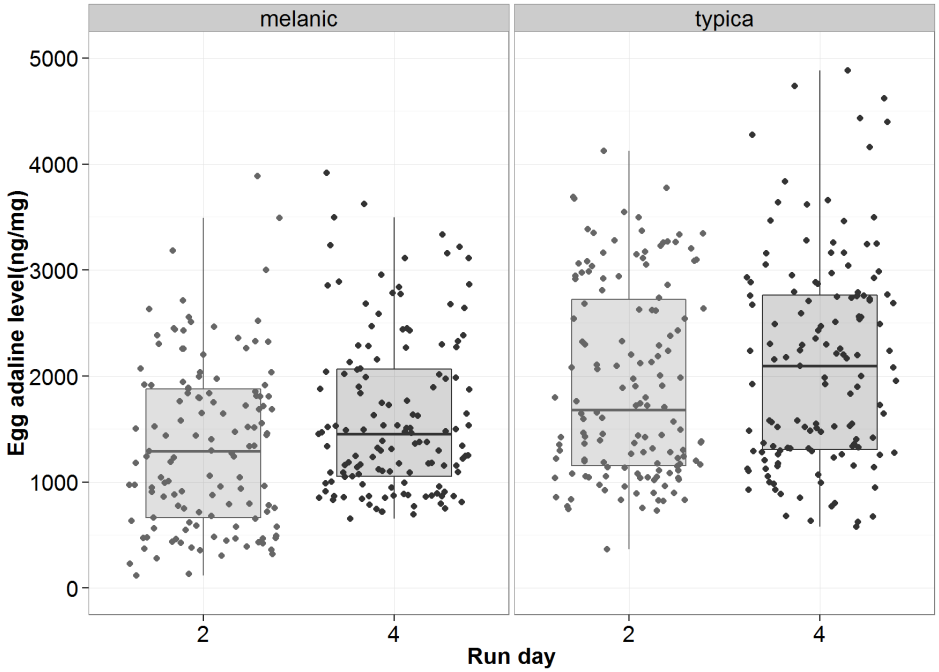

Figure 4. Variation in egg adaline concentration (ng/mg) with female morph (melanic or typica) and run day

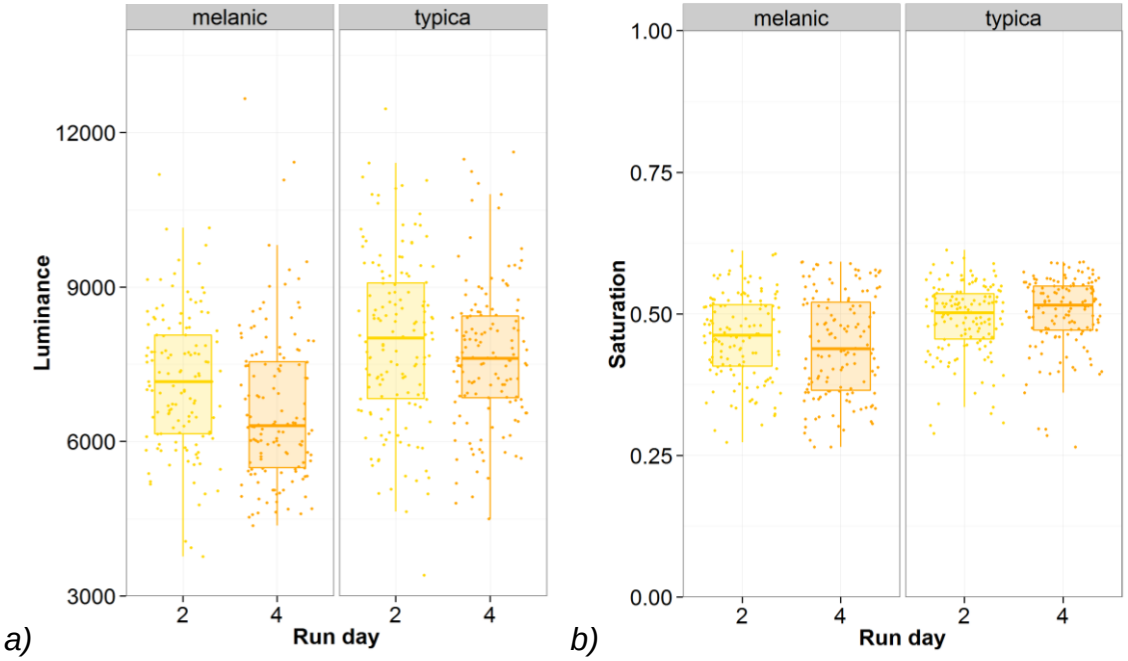

Figure 5. Differences in egg a) luminance and b) saturation between different female morphs (melanic and typical) and experimental run days.

Female laying behaviour

There was a significant interactive effect of treatment and day on the number of females laying eggs (Table 4), though post-hoc tests did not reveal any significant differences between the number of females laying between the first and second halves of the experiment within each of the four treatments. Once the interaction was removed no remaining factors had a significant effect on the likelihood that females would lay eggs. The number of eggs that females laid was significantly affected by both treatment and day, and their interaction (Table 4). In the P+P- and P-P+ treatments, females laid fewer eggs in the half of the experimental period when they were exposed to predator tracks (P+), though this difference was only significant in the P+P- treatment (Figure 6; P+P-: Coefficient=0.65 , Standard Error= 0.18, z value = 3.59, $p<0.01$; P-P+: Coefficient= -0.42, Standard Error= 0.15 , z value = -2.86 , $p=0.07$). There was however no difference in the number of eggs laid in either half of the experiment, in the two control treatments (P+P+ and P-P-; Figure 6). There was no effect of treatment, day, their interaction, female morph or female weight on the maximum size of clusters, whether females laid single eggs or the number of single eggs laid by females (Table 4).

Factor	Females laying eggs		Number of eggs laid		Max cluster size		Females laying single eggs		Number of single eggs laid	
	X_1^2	p	X_1^2	p	X_1^2	p	X_1^2	p	X_1^2	p
Treatment *Day	**13.347**	**0.004**	**24.434**	**0.000**	0.543	0.909	7.397	0.060	0.992	0.803
Treatment	0.068	0.995	/	/	1.520	0.678	0.775	0.856	0.132	0.988
Day	2.288	0.130	/	/	2.048	0.152	0.431	0.511	0.268	0.605
Female morph	0.000	1.000	2.098	0.148	1.968	0.161	1.922	0.166	0.279	0.597
Female weight	0.307	0.579	0.506	0.477	1.363	0.243	0.026	0.873	2.365	0.124

Table 4. Changes in female laying behaviour with treatment, the two halves of the experiment (the first half (run day 2) and the second half (run day 4)), female morph and female weight (egg toxin level also included in all colour metric analyses).

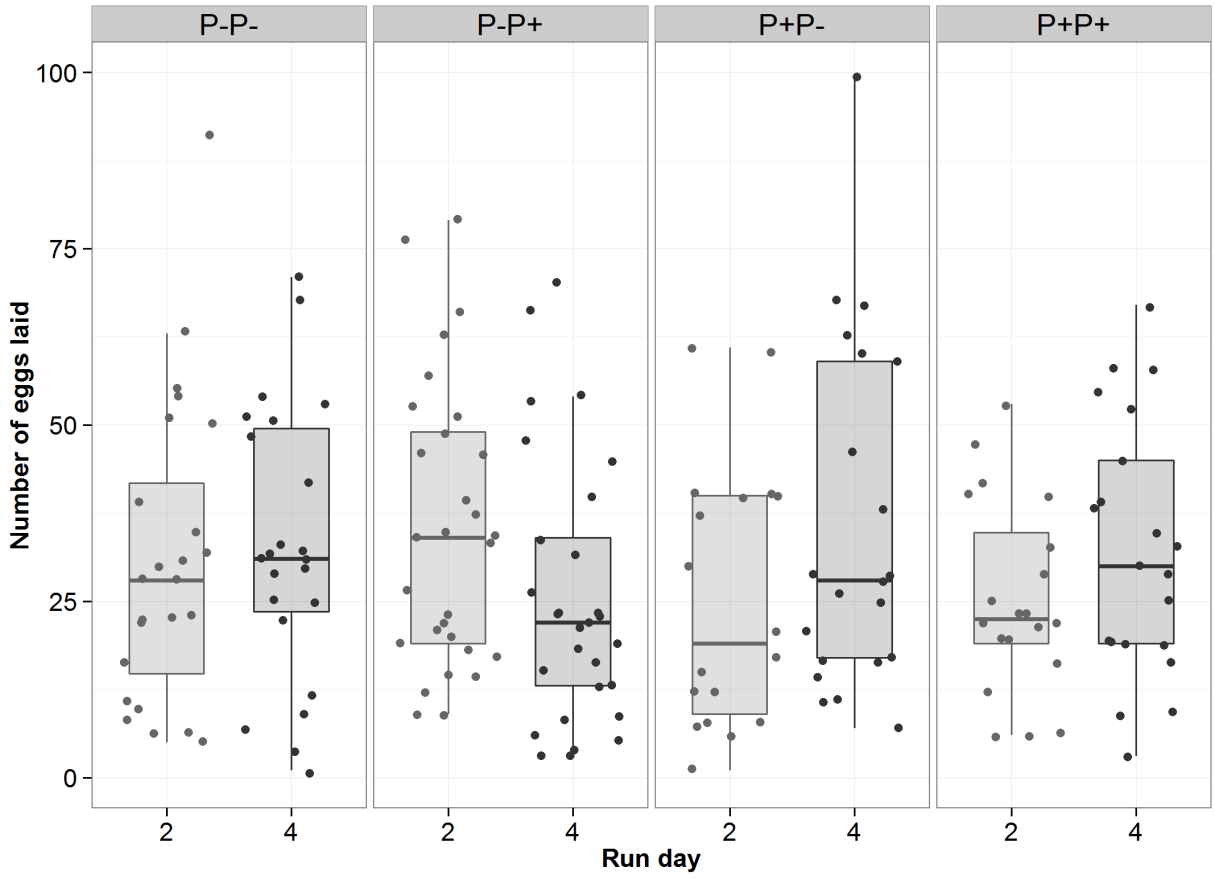

Figure 6.Variation in the number of eggs laid across all treatments and each of the two halves of the experiment, the first half (run day 2) and the second half (run day 4).

Discussion

Female morph had a strong effect on egg phenotype. Contrary to predictions red females laid eggs that had a higher toxin content and greater luminance and saturation values than eggs laid by melanic females. There was an increase in luminance but no increase in egg toxin level in response to increased egg predation risk from *H.axyridis* larvae, for all *A.bipunctata*. Egg toxin level increased over the duration of the experiment, though this increase was smallest when predator tracks were introduced in the second half of the experiment (P-P+ treatment) and greatest when predator tracks were removed (P+P- treatment). The number of eggs laid also increased across the experimental time period in all treatments except P-P+, where the introduction of predator tracks in the second half of the experiment corresponded with a decrease in the number of eggs laid.

Morph differences

Despite differences in appearance between melanic and red *A. bipunctata* adults, females of both morphs lay brightly coloured yellow-orange (aposematic) eggs (Figure 1). However, eggs laid by melanic females had lower values of luminance, saturation, and adaline than those laid by red females, i.e. they were less ‘brightly coloured’ and had lower toxin levels. Maternal influence on offspring toxin level is well documented, and mechanisms include both the direct provisioning of toxins to eggs (Hanifin & Brodie 2003; Kojima & Mori 2015) or to offspring during development (e.g. via trophic eggs Stynoski *et al.* 2014a; Stynoski *et al.* 2014b). Furthermore, a positive correlation between

maternal and offspring coloration in aposematic species has also be found (Winters *et al.* 2014). However, the data presented here provides the first record of differences in the maternal provisioning of aposematic offspring between morphs, with previous studies considering only reproductive output (Nokelainen *et al.* 2012; Dugas & Richards-Zawacki, 2015). There are a number of possible explanations for this difference including; 1) the costs associated with melanism in adults and consequent parent-offspring conflict over maternal resource allocation, 2) pleitropy of genes associated with melanism, and 3) maternal size differences. We expand upon, and compare the relative likelihood of each below.

Costs and trade-offs

The differences in egg phenotype between the two morphs could be a result of the costs associated with melanism. For example, melanic adults are more vulnerable to attack by predators than brightly coloured morphs (Hegna *et al.* 2013; Arenas *et al.* 2015). In ladybird adults a key deterrent of ingestion on attack is the secretion of brightly coloured haemolymph (Marples *et al.* 1994), which contains the defensive toxin that is also found in their eggs (Holloway *et al.* 1991a). This toxin is synthesised within the tissues and in common with endogenously produced toxins in other aposematic species its abundance and concentration is likely limited by associated costs of production and storage (Holloway *et al.* 1991a, b; Blount *et al.* 2009). Moreover, in aposematic species the degree of prey unpalatability dictates the probability of consumption or further attack (Skelhorn & Rowe 2006, 2009). Consequently selection for melanic adults to be able to produce concentrated haemolymph secretions is

likely to be stronger than for red adults, for whom the likelihood of attack is smaller. It therefore also follows that there may be stronger parent-offspring conflict in regards to the allocation of costly toxins, between mothers and offspring of melanic morphs than red morphs, as females balance the trade-off between individual survival and reproductive success (Trivers, 1974).

It is worth noting however, that if the selection pressure to produce toxic defensive secretions was greater for melanic than red females, a disparity between the toxin levels of melanic and red adults might also be expected. Though the toxin level of females in this experiment were not analysed, in a study involving wild collected *A. bipunctata,* toxicity assays did not detect any difference between melanic and red morphs (Arenas *et al.* 2015). The results of biological assays and quantitative analytical chemistry may differ in their sensitivity in detecting fine scale variation in toxin abundance, depending on the toxicity of the chemical compound (Walker, 2014; Speed, 2012). Arguably therefore such assays may potentially not be as sensitive as quantitative analytical techniques in detecting fine scale intraspecific variation in toxin levels, though no direct comparison has yet been made. However, in a randomly selected sample of adults of the two *A.bipunctata* morphs, taken from the same stock culture as the females used in this experiment, no difference in GC quantified toxin levels was detected (Appendix IV).

Such parent-offspring conflict could also arise over carotenoid allocation. Carotenoids act both as pigments, contributing to the coloration of ladybird eggs and elytra (Blount *et al.* 2012; Winters *et al.* 2014), and as antioxidants (McGraw 2005). Also unlike melanin, which is synthesised de novo (Prota 1992;

Sugumaran 2002), they are acquired through the diet (Goodwin 1986). One might assume, as we originally predicted, that there would be greater conflict between red females and her eggs in terms of carotenoid allocation, as the dietary origin of carotenoids limits their availability and these females have a higher signalling demand for carotenoids (Blount et al., 2012). However, melanin is energetically expensive to produce and its production is known to increase the oxidative burden of individuals (Griffith *et al*. 2006). Consequently higher levels of melanism have been linked to the increased mobilisation of antioxidants such as carotenoids (Galvan & Alonso-Alvarez 2008) and a reduction in carotenoid allocation to signals of individual quality (paler red beak and eye rings in red legged partridges; Alonso-Alvarez & Galvan 2011). Therefore there may be trade-offs between maternal carotenoid level and offspring carotenoid level in both morphs.

Accordingly melanic females may have a higher antioxidant, and therefore carotenoid, requirement despite their lack of red warning coloration, resulting in the lower values of luminance and saturation, which correlate with pigmentation levels (Winters *et al*. 2014), in their eggs. The physiological mechanisms regulating melanism production are, however, many and complex (McGraw 2006; Stoehr 2006) it is unclear, for example, whether the consequences of melanin production are relevant only during the pigmentation process, e.g. adult eclosion in ladybird beetles, or whether they have longer term effects (Roulin 2016). A greater understanding of the physiological differences between the two morphs is therefore required in order to elucidate whether such speculation over

the root causes of the differences in egg coloration between the two morphs is valid.

Genetic pleitropy

Alternatively, differences between the eggs of the two morphs may not arise from the *costs* associated with melanism, but through the pleiotropic effects of genes associated with melanistic genotypes (Roulin 2016).The expression of melanistic phenotypes in *A.bipunctata* is under strong genetic control (Lus 1928). Pleiotropic effects could therefore lead to morphs having different life-history strategies (Emaresi *et al.* 2014) and as such investing differentially in their offspring. Following classic reproductive life-history theory one might expect that the lower investment of melanic individuals demonstrated in this experiment would also result in melanics laying a larger number of offspring (Smith & Fretwell 1974). There was no difference between the number of eggs laid by either morph. However, this experiment only looked at a snap shot of maternal investment and such trade-offs are frequently only apparent when considering the life-time reproductive output of an individual (Rollinson & Hutchings 2013). A different pattern may therefore emerge when assessing the life-time reproductive output of each morph.

Female size

It is also worth noting that melanic females were smaller than red females, and the latter may therefore simply have been able to invest more per offspring. Female size has been linked to the production of larger offspring in a number of taxa (Berkeley *et al.* 2004; Hixon *et al.* 2014; Saenz-Agudelo *et al.* 2015)

including ladybirds (Stewart *et al.* 1991), although the latter was a cross species comparison. However, no difference was observed between the morphs in either egg size or mass, which might have been expected if differential investment in eggs based on maternal size had occurred. Nonetheless, there was an increase in toxin level, egg size, and egg mass across the duration of the experiment, independent of female morph. *A.bipunctata,* like many ladybirds, have a triangular fecundity function (Dixon 2000), whereby reproductive investment increases from eclosion until peak fecundity is reached at ~30 days, after which it declines (Lanzoni *et al.* 2004). Females were considerably less than the estimated peak fecundity age (between 15-20 days old) and therefore the increase in egg toxin level and size likely reflects the natural increase in investment of females as they age across the course of the experiment.

Predator treatment

Female *A.bipunctata* did not alter egg toxin content in response to predator tracks, despite the deterrent effects of egg chemical defence on predators (Agarwala & Dixon 1992). This lack of alteration in egg toxin level, in the face of cues of increased egg predation risk, may represent a strategy of reduced investment in order to enable maximum reproduction in more suitable future environments (Philippi & Seger 1989). *H.axyridis* larvae are highly voracious predators (Ware & Majerus 2008; Katsanis *et al.* 2013) and have a greater tolerance of heterospecific toxins than other native ladybird larval predators of *A.bipunctata* (Cottrell 2004, 2007). Therefore reducing investment under these conditions may conceivably increase future reproductive success to a greater

extent than any increase in potential egg unpalatability, by enabling greater future investment in more favourable environments (Jaenike 1978). Such flexible investment strategies are widespread in insects which, like ladybirds, have multiple reproductive bouts (egg laying events) during their lifecycle (Resetarits 1996; Refsnider & Janzen 2010). In accordance with such a hypothesis females in this experiment did lay fewer eggs when in the presence of predator tracks. However, if maternal investment was being selectively reduced then a decrease in egg toxin level and in egg coloration may also have been expected, but these were not seen. Additionally although *H.axyridis* larvae have a greater tolerance of toxins than other species (Katsanis *et al.* 2013), the consumption of heterospecific ladybird eggs (*C.septempunctata* eggs) with a higher toxin content has a greater sublethal effect on their growth and development (Kajita *et al.* 2010). Furthermore, the toxic effects of *A.bipunctata* egg consumption is greater than that of *C.septempunctata* (Sato & Dixon 2004; Sloggett *et al.* 2009), therefore though *H.axyridis* larvae do have higher toxin tolerance, without palatability tests it cannot conclusively be stated that changing egg toxin level would have no or a negligible effect on egg predation.

A more likely explanation is that there may have been costs associated with the alteration of egg toxin level that were not outweighed by the benefits accrued through a possible decrease in egg predation. Such costs could be associated with the concurrent increased risk of sibling cannibalism that accompanies increases in egg toxin level (Chapter 3). Sibling cannibalism is beneficial to maternal fitness when resource availability is low (Perry & Roitberg 2005 a, b) and in *A.bipunctata,* females increase egg toxin level in the absence of aphids,

as this in turn raises the likelihood of cannibalism (Chapter 3). However, in conditions of high resource availability, such as where *ad lib*. aphids are available as in the present experiment, sibling cannibalism is not beneficial. Therefore, there is a risk that increasing egg toxin levels under such conditions, though it may deter predators, might also increase levels of sibling cannibalism in a way that would be maladaptive to maternal fitness (Pfennig 1997), thus constraining maternal-offspring toxin investment.

In contrast to the lack of change in egg toxin level, egg luminance significantly increased in the presence of predator cues. Luminance is considered to be a less important component of aposematic signals than saturation (Arenas *et al.* 2014), however despite this it is known to be important in the detection of prey under low light conditions (Kelber *et al.* 2003), e.g. the underside of leaves where ladybird eggs are laid (Seagraves 2009). Increasing egg luminance may therefore have increased the conspicuousness of eggs and thereby their aposematic signal, or deterrent warning coloration. If egg toxin level was not increased in the face of *H.axyridis* predation because of constraints associated with the maladaptive stimulation of offspring cannibalism, as argued above, it may seem contradictory that a component of egg conspicuousness that signals egg toxin level may at the same time have strengthened. However, the newly emerged larvae responsible for the majority of sibling cannibalism, are likely to differ in their sensory systems from the fourth instar *H.axyridis* larvae, having a more rudimentary visual system (Paulus 1986; Buschbeck 2014). They also differ in their distance from conspecific eggs; siblings will hatch next to them while heterospecific larvae may come across them when foraging (Agarwala &

Dixon 1992; Hironori & Katsuhiro 1997). Chemical as opposed to visual cues may therefore be more important in determining cannibalism by siblings than heterospecific predation. Changes in egg coloration may therefore have less of an impact on the likelihood of sibling cannibalism than egg toxin level.

Consequences for predation

The identified difference in toxicity between melanic and non melanic eggs may result in differential predation risk in the wild, from native heterospecific predators that are known to be susceptible to *A.bipunctata* egg defence toxins (Agarwala & Dixon 1992). However, as stated previously *H.axyridis* larvae are voracious and have a high tolerance of heterospecific toxins, though studies looking at the palatability and toxicity of *A.bipunctata* have produced mixed results (Appendix II; Burgio *et al.* 2002; Sato & Dixon 2004; Ware *et al.* 2009; Katsanis *et al.* 2013). Therefore any change in *A.bipunctata* egg toxicity is likely to have a less pronounced effect on predation by *H.axyridis* than on native predators such as *C.septempunctata* (Katsanis *et al.* 2013). Previous asymmetries in wild predation risk between the eggs of the two morphs, which may in turn have influenced morph abundance, may change with *H.axyridis* invasion. Predation tests using larval *H.axyridis* and other heterospecific ladybird species native to the UK such as *C.septempunctata* are however required to confirm these speculations.

Ladybird eggs also have a variety of other invertebrate predators in addition to larval ladybirds (Smith & Gardiner 2013). These predators are likely to have very different visual systems (Osorio & Vorobyev 2005) and therefore may vary

in their perceptions of differences between eggs of different morphs and eggs laid under other conditions (Stevens 2007). Insects are also strongly chemically motivated (Blomquist & Bagneres 2010) and ladybirds are no exception (Wheeler & Carde 2013; Wheeler *et al.* 2015); eggs may therefore signal their toxicity not only through colour but through chemical signals such as hydrocarbons ('chemical aposematism' Weldon 2013). Egg species identity has, for example, been shown to be detected via chemicals on the exterior of ladybird eggs, which in turn affects predation risk (Hemptinne *et al.* 2000b). Consequently though we have revealed some of the causes and discussed possible consequences of toxin variation on ladybird egg predation risk, a wider view encompassing a greater number of predators and sensory modalities is required to reveal the full picture.

Summary

In conclusion we provide the first demonstration that the level of offspring chemical defence and associated warning coloration differ between morphs of an aposematic species. Eggs of both morphs signal their toxin level honestly. The lower luminance, saturation and toxin level of eggs laid by melanic females may result from either a) the associated costs of melanism for mothers or b) the pleiotropic effects of melanisitic genes. Differing toxicity levels indicate that melanic eggs, like melanic adults, may be more susceptible to predation from native predators, but the persistence of melanic morphs within populations suggests that this is offset by other positive selection pressures. The response of females to larval *H.axyridis* cues, suggests that eggs of *A.bipunctata* are at risk of predation by the invasive *H.axyridis*. Predation tests using ladybird and

other larval predators are required to confirm the degree to which predation of the eggs of each morph by native predators is asymmetrical and how this asymmetry will change in the light of the spread of the invasive *H.axyridis* in the UK.

CHAPTER 5

Predation risk and parental effects influence the toxicity and colour of ladybird eggs

Abstract

In species that advertise their toxicity to predators through visual signals, there is considerable variation among individuals in both signal appearance and levels of defence. Parental effects, i.e. non-genetic inheritance, may play a key role in creating and maintaining this diversity, however a comprehensive test of this notion is lacking. Using the ladybird *Adalia bipunctata* we assess whether egg coloration and toxin level (concentration of the toxic alkaloid adaline) is influenced by maternal perception of offspring predation risk, while also considering the effect of parental phenotype. We show that egg coloration, but not egg toxin level, varies in response to predation risk, and that the direction of this change is dependent upon predator species identity. Egg luminance (lightness) decreases in response to conspecific but not heterospecific predation risk, while conversely egg saturation increases in response to heterospecific but not conspecific predation risk. Furthermore, maternal toxin level and paternal elytral coloration positively predicted egg toxin level and egg coloration, respectively. This study provides the first demonstration of maternally mediated offspring colour change in response to predation risk and highlights the importance of studying multiple non-genetic parental effects in determining offspring phenotype.

Introduction

In some species, protection from predators is gained through the association of a colourful warning signal with a toxic or distasteful defence (aposematism; Poulton 1890; Ruxton *et al.* 2004). Individuals within a species may therefore benefit from sharing similar levels of defence and conspicuousness (Rowland *et al.* 2010). Despite this, considerable variation in signal expression and associated toxin level is found between individuals of the same aposematic species (Summers *et al.* 2003; Cortesi & Cheney 2010; Blount *et al.* 2012; Manuel Vidal-Cordero *et al.* 2012). A number of hypotheses have recently been proposed which help to explain how such apparently paradoxical variation may still be part of an evolutionarily stable strategy, yet these focus almost exclusively on adult phenotypes (reviewed in Speed *et al.* 2012; and Summers *et al.* 2015). Natural selection, however, acts at every stage of an organism's life cycle (Stearns 1992) with both the strength and nature of selection pressures varying accordingly to life stage (Moran 1992). This is especially relevant for aposematic species, the majority of which have complex lifecycles where, for example, each discrete phase (i.e. egg, larval, or adult) is likely to have very different predators e.g. (Hemptinne *et al.* 2012). Furthermore, not only is offspring phenotype key in determining which individuals survive to contribute to the adult population, but many aspects of offspring phenotype carry over into adulthood (Monaghan 2008; Burton & Metcalfe 2014). It is clear therefore, that to fully understand the variation observed in adult phenotype in aposematic species, consideration of offspring phenotype and the factors that determine it is required (Day & Bonduriansky 2011; Marshall & Morgan 2011).

In addition to their genetic contribution, the phenotype of parents plays a key role in regulating offspring phenotype (‘non-genetic inheritance’ Marshall & Uller 2007; Bonduriansky & Day 2009). Known as parental effects, this alteration of offspring phenotype by the parental phenotype occurs via a number of mechanisms including the transfer of hormones (Groothuis *et al.* 2005), macronutrients (Royle *et al.* 1999) and micronutrients (e.g. compounds with antioxidant capacities (Blount *et al.* 2000)), antibodies (Boots & Roberts 2012), and defence chemicals (Winters *et al.* 2014b). Due to the typically larger per offspring investment of mothers, maternal effects are generally considered to be a comparatively greater determinant of offspring phenotype than paternal effects (Waage,1997; Crean & Bonduriansky, 2014) and are posited to have played a role on the evolution of aposematism itself (Brodie & Agrawal 2001). For example, in chemically defended species maternal toxin level is often correlated with offspring toxin level, even when that toxin is sequestered from the environment (Bezzerides *et al.* 2004; Kojima & Mori 2015). Furthermore, in aposematic species components of maternal and offspring warning colouration, in addition to toxin levels, are also positively correlated (Winters et al. 2014). This type of maternal effect, where maternal and offspring phenotype correlate, is predicted to arise in traits that experience strong selection, when there is environmental autocorrelation between generations (Hoyle & Ezard 2012; Kuijper & Hoyle 2015).

Maternal effects are, however, not simply restricted to the non-genetic inheritance of the maternal phenotype, they also encompass powerful mechanisms whereby females can alter offspring phenotype in response to

variation in the offspring environmnet (Fox *et al.* 1997; Agrawal 2001; Galloway & Etterson 2007) and to mate phenotype or 'quality' (Burley 1988; Gowaty 2008). For example, the maternal detection of predators or predator cues results in offspring with phenotypic characteristics that enhance their avoidance and evasion of predators (Storm & Lima 2010; Coslovsky & Richner 2011; Giesing *et al.* 2011; Bestion *et al.* 2014). Moreover, females have been observed either increasing (Sheldon 2000; Horvathova *et al.* 2012) or decreasing (Saino *et al.* 2002; Bolund *et al.* 2009) their investment in offspring, in response to male attractiveness (Ratikainen & Kokko 2010). This variation in investment in turn affects offspring phenotype, for example female zebra finch (*Taeniopygia guttata*) that mate with more attractive males, i.e. those with a large colourful ornament, in turn produce sons with larger ornaments (Tschirren *et al.* 2012).

Such adaptive maternal effects enable mothers to fine tune their offspring investment per reproductive event, maximising the total number of surviving offspring (reproductive success) and thereby maternal fitness (Smith & Fretwell 1974; Bernardo 1996). This is likely to be selectively beneficial when differential investment carries associated costs, e.g. the chemical defence and associated warning signals of aposematic species (Higginson *et al.* 2011; Lindstedt *et al.* 2011). The maternal alteration of offspring colour in response to environmental variation is known to occur in non-aposematic species (Abram *et al.* 2015) and within aposematic species themselves warning signals can function as sexual signals (Summers *et al.* 1999), with male warning signal strength dictating female preference (Maan & Cummings 2008; Finkbeiner *et al.* 2014). However,

as yet no association between either offspring predation risk or male warning signal strength and maternal investment in both offspring defence and colouration has been demonstrated in any aposematic species. Here we investigate how female reproductive investment, and therefore offspring phenotype, changes in response to increased levels of offspring predation risk, while simultaneously accounting for the effects of both female and male phenotype, in the aposematic ladybird *Adalia bipunctata*. We focus specifically on egg phenotype as, in species such as *A. bipunctata* where there is no parental care, egg provisioning is the predominant mechanism via which non-genetic parental effects occur (Newcombe *et al.* 2015).

A. bipunctata eggs are aposematic and laid in environments with high levels of predation from the larvae of ladybird competitors (intraguild predation, Polis *et al.* 1989). Egg toxins deter heterospecific predators, but attract conspecific cannibals (Kajita *et al.* 2010; Chapter 3). Gravid females can alter egg laying behaviour in response to chemical cues of offspring predators (Seagraves 2009). Moreover they increase egg toxin level in the absence of aphids, a strategy hypothesised to increase levels of sibling cannibalism under conditions where it benefits maternal fitness (Chapter 3). However, whether they also alter egg toxin level and conspicuousness in response to conspecific or native heterospecific predatory larvae is unknown as to date only egg toxin changes in response to invasive species have been investigated (Paul *et al.* 2015). In this case the lack of change in egg toxin level observed in the presence of larval tracks of the invasive *Harmonia axyridis,* is thought to be a consequence of

constraint; the increased number of eggs laid in the presence of *Harmonia axyridis cues* trading off with egg toxin level (Chapters 2 & 3).

Here we test the egg laying response, specifically changes in egg toxin level and egg coloration of female *A. bipunctata* to the larval tracks of *Coccinella septempunctata* (a native heterospecific ladybird egg predator) and to the tracks of unrelated conspecific larvae. We predict: 1) in the presence of conspecific (*A. bipunctata*) larval tracks egg toxin level will decrease and egg coloration will change in order to decrease egg cannibalism risk; 2) in the presence of heterospecific (*C. septempunctata*) larval tracks egg toxin level will not change but egg coloration will vary in a way that strengthens the egg's aposematic signal; and 3) both egg toxin level and egg coloration will positively correlate with maternal toxin level and elytral coloration (Winters *et al.* 2014), and 4) with paternal toxin level (Camarano *et al.* 2009) and elytral coloration, as predicted by female responses to colourful signals of male quality (Maan & Cummings 2008; Finkbeiner *et al.* 2014).

Methods

Culture and experimental set up

Stock culture of *A. bipunctata* (*typica*), obtained from Gardening Naturally (Love Lane Industrial Estate, Cirencester, UK), were maintained in culture on an *ad lib.* diet of pea aphids (*Acyrthosiphon pisum*) [*A. pisum* reared on dwarf bean (*Vicia faba*) Sutton variety] at 18^{o}C with a 16L:8Dh photoperiod. Experimental individuals were 1st generation virgin adults of known age ($\bar{x}$ = 21 days post

eclosion) obtained from stock culture: 104 females and 104 males from 20 families. Females were mated with a non-sib male, and 24 h after pairing males were removed, photographed, and stored at -80^{o} C prior to toxin analysis (see below for colour and toxin analysis method details). Females were then placed into a clean petri dish with adlib aphids (0.01g, ~ 40 aphids; Hodek *et al.* 2012)). After a further 24 h a cluster of eggs was randomly selected from those laid by the females and a further subset of 3 eggs from the cluster were photographed and stored at -80^{o} C. Females were then weighed to nearest 0.01 mg (analytical balance GR-200 A&D® Gemini™) and placed into an individual experimental arena, in one of three treatments (control (NN), conspecific risk (CON), or heterospecific risk (HET), with an ad lib aphid supply. Females from different sibling clusters were distributed evenly between the treatment levels, so that family ID and mate ID were represented equally in all three treatments (NN: n= 41, CON: n=41, HET: n= 22). The simulated predation risk treatment levels were created using tracks of either 4th instar *A. bipunctata* larvae (CON) or *C. septempunctata* larvae (HET). For each replicate, tracks were created using 5 larvae, which were placed, without food, into individual sterile Petri dishes (9 cm diam.), each containing a semicircle of corrugated filter paper (9 cm diam.) and left for 24 h (Doumbia *et al.* 1998; Magro *et al.* 2007). The control environment of no simulated predation risk (NN) consisted of a sterile Petri dish (9 cm diam.) and a clean semicircle of corrugated filter paper that had not been in contact with any ladybird larvae. Each female was left in its experimental arena for 2 d (48 h), with additional aphids added at 24 h. Laying behaviour was monitored at 1, 3, 6, 9, and 24 h intervals over the 2 d, with egg number, cluster size, and the

number of clusters laid recorded. A cluster was classified as a group of two or more eggs, with each egg being in physical contact with at least one other egg in that cluster. Once recorded, eggs were removed using a damp paintbrush, to prevent cannibalism. The first and last clusters laid by a subset of females (NN: n= 19, CON: n=13, HET: n= 15) over the 2 d were chosen, and a further subset of 3 eggs from each cluster photographed and stored at -80^{o}C prior to toxin analysis. After the full 3 days of the experiment had elapsed, females were removed, photographed and stored at -80^{o}C prior to toxin analysis. All experiments were carried out in an incubator (Percival® model I-41LL, 505 Research Drive, Perry, IA 50220 USA) at 18^{o}C with a 16L:8D h photoperiod.

Quantifying egg colour

Digital image analysis and visual modelling were both used to quantify egg and elytral luminance (perceived lightness) and saturation (colour richness) (Kelber *et al.* 2003; Osorio & Vorobyev 2005). Individual eggs and adults were photographed using a Nikon D7000 digital camera which had undergone a quartz conversion, enabling ultraviolet (UV) light sensitivity (Advanced Camera Services, Norfolk, UK), fitted with a Nikon 105-mm Nikkor lens. For photographs in the visible spectrum the camera was fitted with an ultraviolet (UV) and infrared (IR) blocking filter (Baader Planetarium, Mammendorf, Germany UV/IR Cut filter; transmitting between 400 and 700 nm). For photographs in the UV part of the spectrum the camera was fitted with a UV pass IR blocking filter (Baader U filter; transmitting between 300 and 400 nm). All photographs were taken in a dark room using standardized lighting provided by a UV daylight lamp (Iwasaki eyeColor arc lamp (6500k), with UV coating removed) with eggs or

adults placed on a sheet of black ethylene-vinyl acetate (EVA), used for its low (<5%) UV reflectance, next to a Spectralon™ 40% diffuse grey reflectance standard (Labsphere, Congleton, UK) (Stevens *et al.* 2007; Arenas et al, 2014).

To correct for the non-linear response of the camera to light levels (radiance), and for any variation in light levels between photos, each image was linearized with respect to light intensity and equalized with respect to the grey standard (Stevens *et al.* 2007). This was carried out using the programme ImageJ 1.47t and the Multispectral Image Calibration and Analysis Toolbox plugin (Troscianko & Stevens, 2015). The entirety of each egg was selected for analysis, using a specialised egg selection tool plugin (Troscianko 2014), and for adults an area of the elytra with no spectral reflectance and excluding the achromatic spots, was selected (Arenas *et al.* 2015). It is important when investigating any changes in anti-predator coloration to do so in the context of predator vision (Endler 1978; Endler & Mielke 2005; Stevens 2007). Using the image transformation approach developed in Troscianko & Stevens (2015), linearized egg images were mapped to the predicted responses of the ladybird visual system (Cottrell 2007), using the spectral sensitivity of the ladybird *C. septempunctata* (Lin *et al.* 1992) and linearized images of adult elytra were mapped to the predicted responses of an avian visual system (Marples *et al.* 1989), using blue tit (*Cyanistes caeruleus*) spectral sensitivities (Hart *et al.* 2000). This mapping technique is highly accurate compared to modelling photon catch data with reflectance spectra (Stevens *et al.* 2014; Troscianko & Stevens, 2015).

Luminance is here used to refer to a visual system dependent measure of achromatic variation (Osorio & Vorobyev 2005). For the measures of adult elytra modelled using blue tit vision, luminance is measured using the values obtained for double cones. Ladybird vision is potentially trichromatic, containing three classes of retinular cells sensitive to mediumwave (MW), shortwave (SW) and ultraviolet (UV) light (Lin et al. 1992). The receptor type used for luminance vision varies considerably between different insects, but is generally the most abundant of the retinular cell classes (Osorio & Vorobyev 2005). In ladybirds this is the MW channel (~520 nm), with six MW receptors to each one of the other two receptor types per adult ommatidium (Lin et al. 1992), and as such this receptor was used for luminance calculations for data extracted using ladybird vision. Saturation is used as a measure of chromatic variation, specifically here it is a visual system dependent measure of the richness of a colour and how it differs from neutral grey or white (Kelber *et al.* 2003). Prior to calculations of saturation, UV, SW, MW, and LW cone catch values were converted into proportions to remove absolute variation in brightness (Endler & Mielke 2005). The proportional cone catch values were then converted into three colour space coordinates (X, Y, Z), giving each individual a location of colour in three dimensional colour space (Endler & Mielke, 2005; Kelber *et al.* 2003). Saturation was calculated as the shortest Euclidian distance from the achromatic origin, with saturation being greater the further a point is from the origin. Due to strong correlation with both luminance and saturation (Appendix IV.) measures of hue were not included in the analysis (Arenas *et al.* 2015; Chapter 4).

Quantifying levels of adaline

A. bipunctata eggs and adults contain the toxic alkaloid adaline; this was assayed as follows. Each egg was weighed to the nearest 0.1 µg using an electronic microbalance (Cahn C33; Scientific and Medical Products Ltd, Manchester, UK.) and homogenized for 30 seconds in 200 µl of dichloromethane (DCM), using a handheld electronic pestle. Each sample was then centrifuged at 13RPM and 4^{o} C for 10 minutes. 100 µl of solution was transferred into a screw top autosampler vial. Adults (male and females) were weighed to the nearest 0.01 mg (analytical balance GR-200 A&D® Gemini™), elytra removed (as it is the soft tissue that contains the adaline (Laurent *et al.* 2002) and placed into a 2 ml centrifuge tube along with 1 ml of DCM and 0.5 ml of glass beads (1 mm diameter). Samples were homogenised for 1 min at 5.5m/s in a tissue homogenizer (Precellys 24 Tissue Homogenizer; Bertin Technologies, France) and centrifuged at 13RPM and 4^{o}C for 10 minutes. 100µl of supernatant from the resulting solution was transferred into a screw top autosampler vial along with 900µl of DCM. Samples (2µl) were analysed on a non-polar (HP-1, 50 m x 0.32 mm inner diameter x 0.5) Gas-Chromatograph (GC) (Agilent Technologies, UK) fitted with a cool-on-column injector, a deactivated HP-1 pre-column (1m x 0.53 mm inner diameter) and a flame ionisation detector (FID). The GC oven temperature was maintained at 30°C for 1 min after sample injection and then raised by 5°C min^{-1} to 150°C, then 10°C min^{-1} to 240°C. The carrier gas was hydrogen. Peak enhancement by co-injection with a pure adaline standard was used to confirm correct identification of the adaline peak. Absolute adaline concentration per egg (ng/mg) was

quantified by transforming the peak area using a calibration curve created from an external standard of pure adaline in dichloromethane of the following concentrations; 100ng/µl, 50ng/µl, 10ng/µl, 5ng/µl, and 1ng/µl.

Data analyses

Data were analysed using R version 3.0.1(R Core Team, 2015). Where appropriate, data were examined for normality, homoscedasticity and outliers. Alpha level was set at 0.05 for all tests and stepwise backwards deletion was employed to reach the minimum adequate model (Crawley, 2013). General or generalised linear models (package=MASS) were used to assess the effect of treatment, female mass (mg) and female age (days) on the time the first eggs were laid (family= negative binomial), the number and maximum size of clusters (family= negative binomial), the total number of eggs (family = Gaussian), and whether or not females laid single eggs (family= binomial). Egg volume (mm^3), adaline levels (ng/mg), luminance, and saturation were repeatable (Egg volume: R = 0.723, SE= 0.064, CI= [0.584, 0.822], P<0.001; Egg adaline: R = 0.935, SE= 0.019, CI= [0.886, 0.961], P<0.001; Luminance: R = 0.849, SE=0.037, CI= [0.761, 0.904], P<0.001; Saturation: R = 0.419, SE=0.096, CI= [0.216, 0.592], P<0.001 (Nakagawa & Schielzeth 2010; 'rptr' package Nakagawa & Schielzeth 2013)). The effect of treatment, day, treatment by day interaction, female and male adaline level (ng/mg) or mass (mg), and female age on the square root of egg adaline level (transformed to normalise) or egg volume were assessed using a general linear mixed effects model (LMER; package=lme4 (Bates *et al.* 2015)), where female identity was the random effect. To investigate whether

egg luminance and egg saturation correlated with egg adaline levels and whether this relationship varied between treatments a LMER was used. Variation in egg luminance and egg saturation attributable to the effect of egg adaline level (ng/mg), treatment, day, egg adaline level (ng/mg) by treatment interaction, egg adaline level (ng/mg) by day interaction, treatment by day interaction, and maternal/paternal luminance/saturation was ascertained with female identity was the random effect. The relationship between parental adaline levels (ng/mg) and both elytral luminance and elytral saturation was tested using a generalised linear model (family = negative binomial) and general linear model (family=Gaussian) respectively for both females and males, while also controlling for mass (mg).

Results

Response to conspecific (A. bipunctata) and heterospecific (C. septempunctata) tracks

Predator treatment had a significant effect on the latency of females to lay eggs and both the total number of clusters and eggs laid (Table 1). Females took the longest to lay eggs in the presence of conspecific tracks. control (NN) – conspecific (CP): mean difference $\pm$SE = 0.33$\pm$0.15hrs, $Z_{2,101} = -2.17$, $p = 0.03$; heterospecific (HP) – conspecific (CP): mean difference $\pm$SE = 0.48$\pm$0.19hrs, $Z_{2,101} = -2.53$, $p = 0.01$) and also laid both fewer clusters and a smaller total number of eggs than the control treatment (Cluster number: mean difference $\pm$SE = 0.25$\pm$0.0.12, $Z_{2,103} = 2.07$, $p = 0.04$; total egg number: mean difference

±SE =-10.03±2.80, $t_{2,103}$ = -3.56, p <0.001). There was a trend for females to lay smaller clusters in the presence of heterospecific offspring predator tracks (mean difference ±SE =-0.36±0.15865, $z_{2,103}$= -2.282, p= 0.02), though overall the effect of treatment on both average and maximum cluster size was non-significant (Table 1)

Table 1. Effect of predation risk (conspecific and heterospecific) and female age and mass on female laying behaviour. Results are given as, d.f., test statistic, and p-value.

Factor	d.f.	Latency to lay eggs		Number of clusters		Maximum cluster size		Average cluster size		Total Eggs		Single eggs laid		Total number of single eggs	
		X^2	p	X^2	p	X^2	p	X_1^2	p	F	p	X_1^2	p	F	p
Treatment	**2**	**8.22**	**0.02**	**6.78**	**0.03**	**5.34**	**0.07**	2.38	0.10	**6.49**	**<0.01**	4.59	0.10	3.45	0.18
Female mass (mg)	**1**	0.00	0.95	**13.63**	**0.00**	1.92	0.17	0.13	0.72	**35.93**	**<0.01**	1.89	0.17	1.68	0.20
Female age (days)	**1**	0.02	0.90	0.69	0.41	1.40	0.24	0.61	0.44	**6.71**	**0.01**	1.26	0.26	0.28	0.60

Table 2. Effect of predation risk (conspecific and heterospecific), experimental day, their interaction, and the effects of paternal values of adaline or paternal mass on egg adaline and egg volume respectively . Results are given as, d.f., test statistic, and p-value.

Factor	d.f.	Egg adaline (ng/mg)		Egg volume (mm^3)	
		X_1^2	p	X_1^2	p
Treatment	2	0.52	0.77	2.38	0.30
Day	2	**21.76**	**<0.001**	**6.54**	**0.04**
Treatment*Day	4	3.25	0.52	6.55	0.16
Maternal adaline/mass	1	**14.93**	**<0.001**	0.66	0.42
Paternal adaline/mass	1	1.01	0.31	0.40	0.53
Maternal adaline/mass * Paternal adaline/mass	1	0.12	0.73	0.22	0.64

There was no interactive effect of treatment and day on egg adaline levels (Table 2). However, adaline levels significantly decreased from day 0 (Figure 1; Day 0-Day 1, mean difference $\pm$SE =-3.10$\pm$0.96, $Z_{2,103}$= -3.10, p<0.01; Day 0-Day 2, mean difference $\pm$SE =-4.75$\pm$1.01, $Z_{2,103}$= -4.68, p<0.001). Irrespective of treatment, egg volume also decreased between days 0 and 1 (Table 2: mean difference $\pm$SE = 0.01$\pm$0.004, $t_{2,103=}$2.56, p=0.03). There was an interactive effect of treatment and day on both egg luminance and egg saturation (Table 3). In the presence of conspecific predator tracks (CP) egg luminance decreased once females were exposed to tracks (Figure 2). This trend was significantly different to both the control (NN) and heterospecific predator treatment (HP) (Luminance NN-CP t value = 3.58, HP-CP t-value = 3.63, NN-SP t-value = 0.19 (NS)). No statistically significant change in egg saturation was observed under (CP), though a decreasing trend was observed (Figure 3), however there was an increase in egg saturation over the days in the heterospecific predator treatment (Saturation NN-CP t value = 0.89 (NS), HP-CP t-value = 2.82, NN-HP t-value = 1.94). Overall, egg luminance and saturation both correlated positively with egg adaline concentration (ng/mg) (Figures 4 & 5, Table 3). However the relationship between egg luminance and egg adaline changed when females were exposed to conspecific predator tracks (CP) (Figure 4; Table 3).

Table 3. Variation in egg luminance and saturation with egg adaline concentration, predation treatment, experimental day, and there interactive effects and with parental values of either luminance or saturation respectively. Results are given as, d.f., test statistic, and p-value.

Factor	d.f.	Egg luminance		Egg saturation	
		X^2	p	X^2	p
Treatment	2	/	/	/	/
Day	1	/	/	/	/
ng.mg	**1**	/	/	**5.05**	**0.025**
Treatment*Day	**2**	**17.85**	**< 0.01**	**8.11**	**0.017**
ng.mg*Treatment	**2**	**14.52**	**< 0.001**	3.58	0.17
ng.mg*Day	**2**	**31.57**	**< 0.001**	0.00	0.99
ng.mg*Treatment*Day	4	3.22	0.20	3.54	0.17
Maternal Lum/Sat	1	0.52	0.47	0.00	0.96
Paternal Lum/Sat	**1**	**12.33**	**< 0.001**	**3.23**	***0.07***
Maternal Lum/Sat * Paternal Lum/Sat	1	0.46	0.50	1.52	0.22

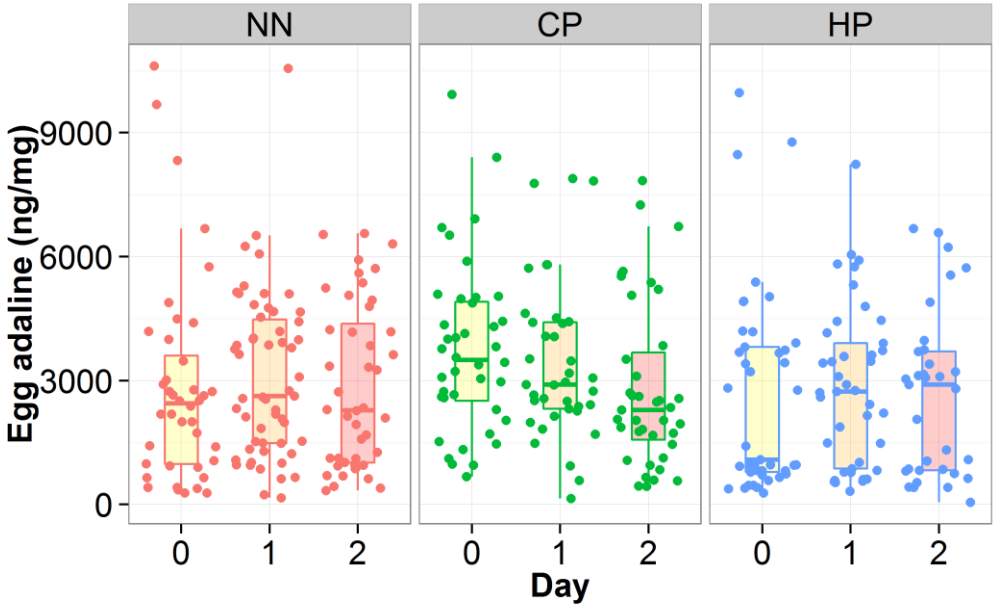

Figure 1. Concentration of adaline (ng/mg) in *A.bipunctata* eggs laid in the absence of offspring predator tracks (NN - red), or the presence of either conspecific offspring predator (CP - green) or heterospecific offspring predator (HP - blue) tracks.

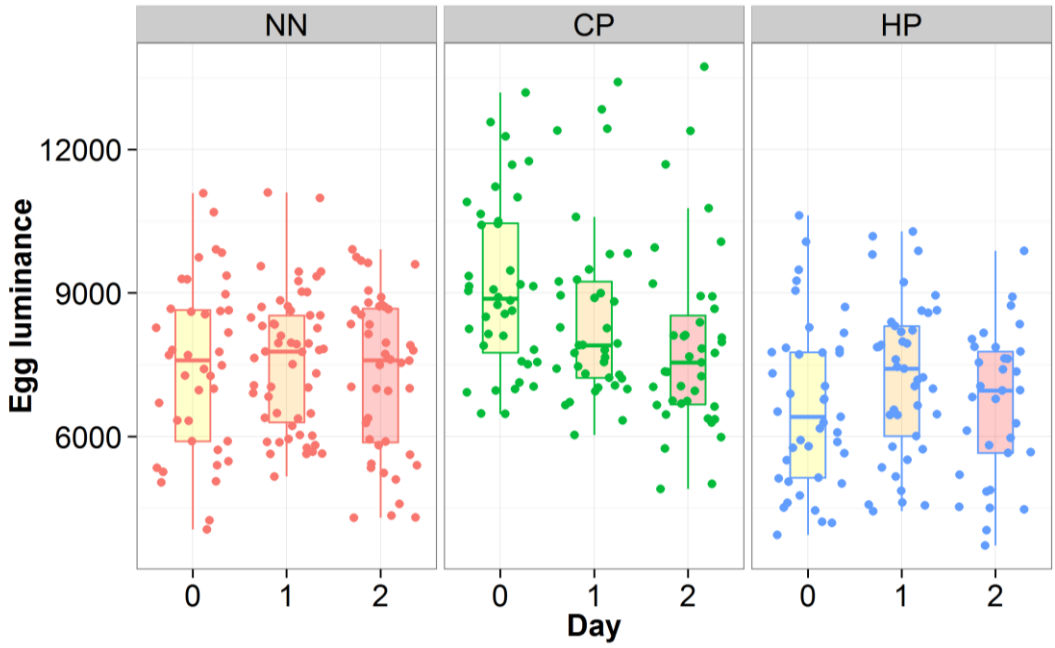

Figure 2. Luminance of *A.bipunctata* eggs laid in the absence of offspring predator tracks (NN - red), or the presence of either conspecific offspring predator (CP - green) or heterospecific offspring predator (HP - blue) tracks.

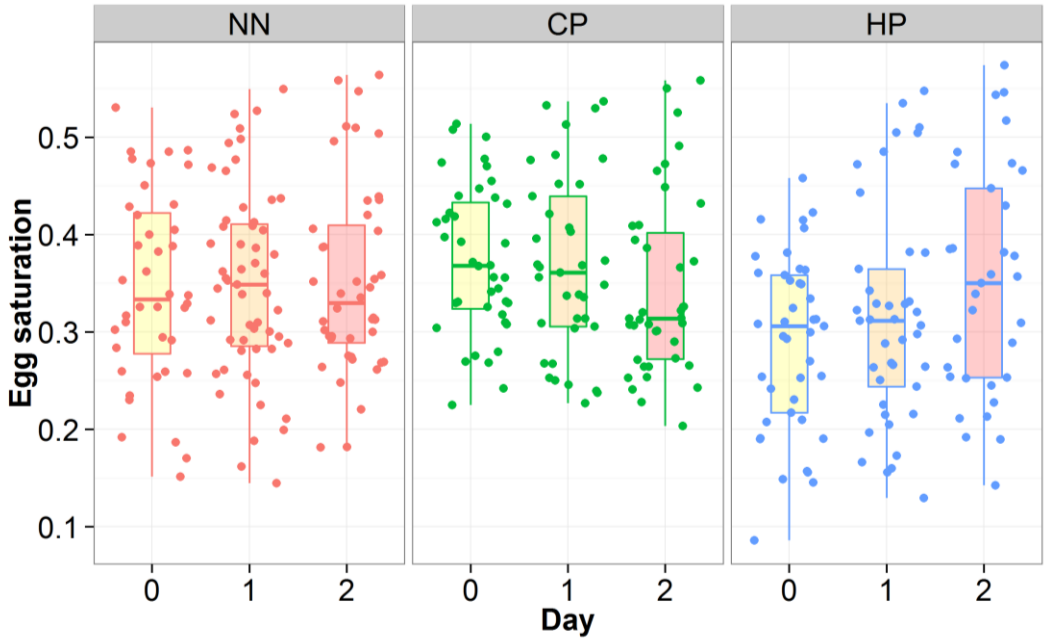

Figure 3. Saturation of *A.bipunctata* eggs laid in the absence of offspring predator tracks (NN - red), or the presence of either conspecific offspring predator (CP - green) or heterospecific offspring predator (HP - blue) tracks.

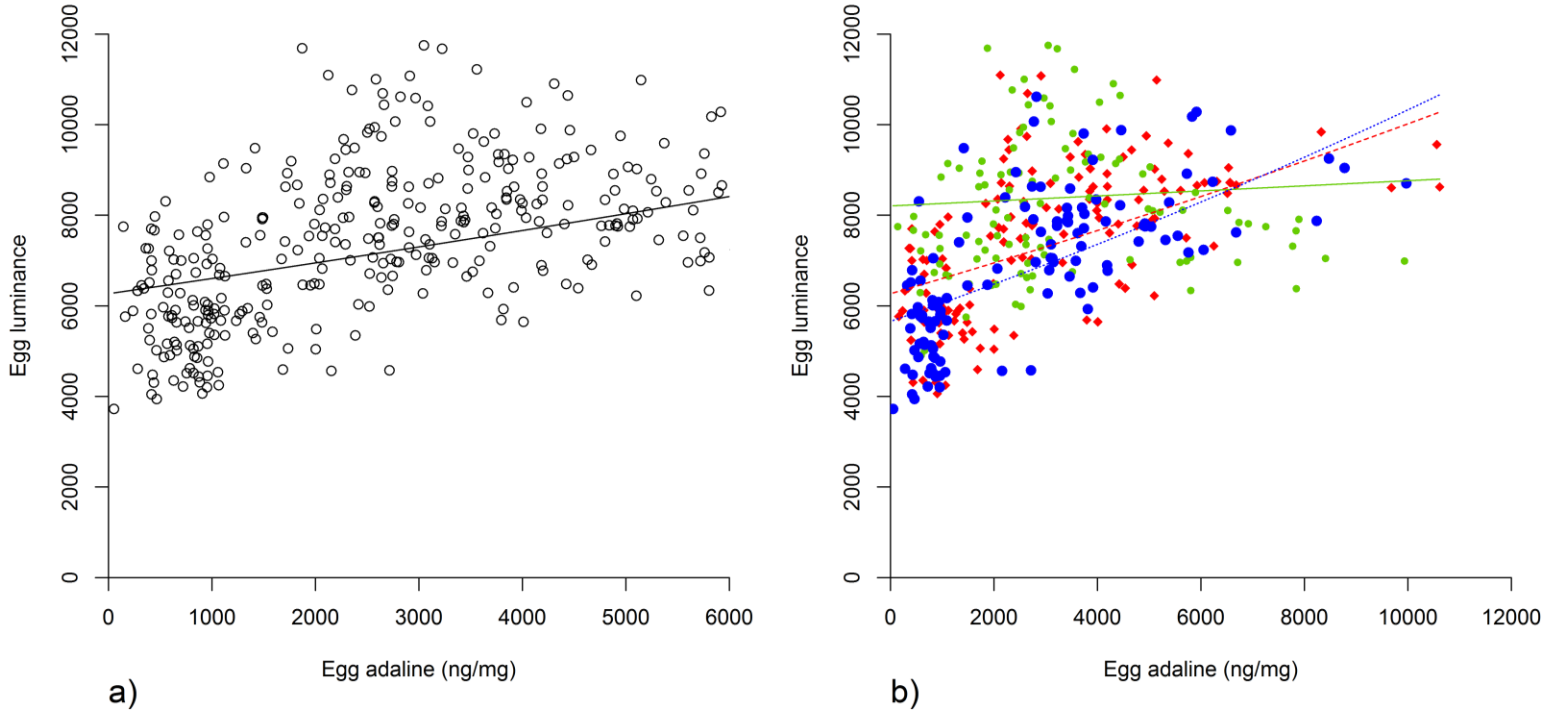

Figure 4. Relationship between a) egg luminance and egg adaline concentration and b) how this relationship differs depending on treatment; absence of offspring predator tracks (NN - red), presence of conspecific offspring predator (CP - green) or heterospecific offspring predator (HP - blue). Trend lines are glmm model predictions (see Methods for model details).

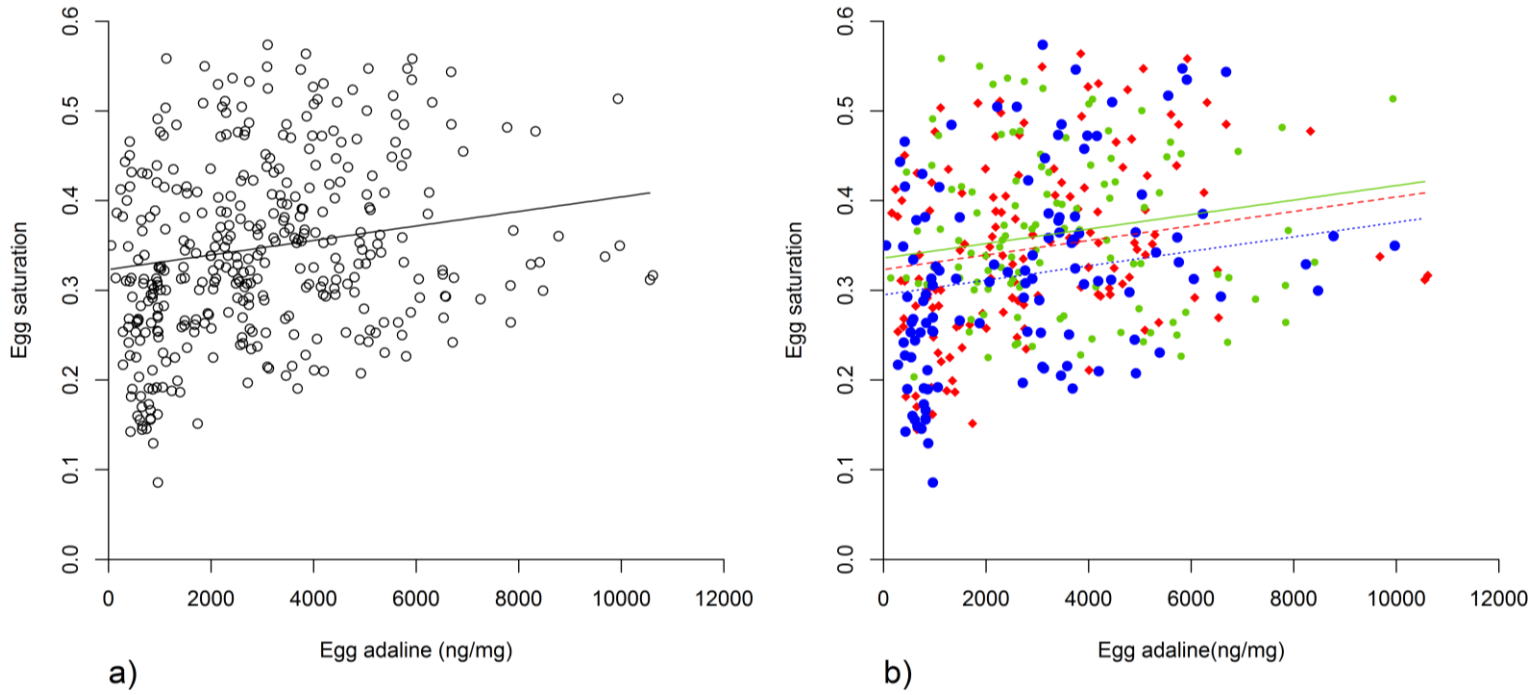

Figure 5. Relationship between a) egg saturation and egg adaline concentration and b) how this relationship does not differ with treatment; absence of offspring predator tracks (NN - red), presence of conspecific offspring predator (CP - green) or heterospecific offspring predator (HP - blue). Trend lines are glmm model predictions (see Methods for model details).

Maternal and paternal investment in offspring

Independent of male adaline levels, maternal adaline concentration was positively correlated with egg adaline concentration (Figure 6, Table 2) but neither measure of female elytral colour was correlated with egg colour (Table 3) There was a significant positive correlation between egg luminance and paternal elytral luminance (Figure 7, Table 3) and a positive trend between egg saturation and paternal elytral saturation (Figure 8, Table 3). Adult elytral saturation was positively correlated with adaline level in females ($F_{1,47}$=8.61, p<0.01) but not in males ($F_{1,47}$=8.61, p=0.86) and elytral luminance showed no relationship with adaline level in either sex (females: $F_{1,48}$=0.01, p=0.93; males: $F_{1,48}$=0.01, p=0.93).

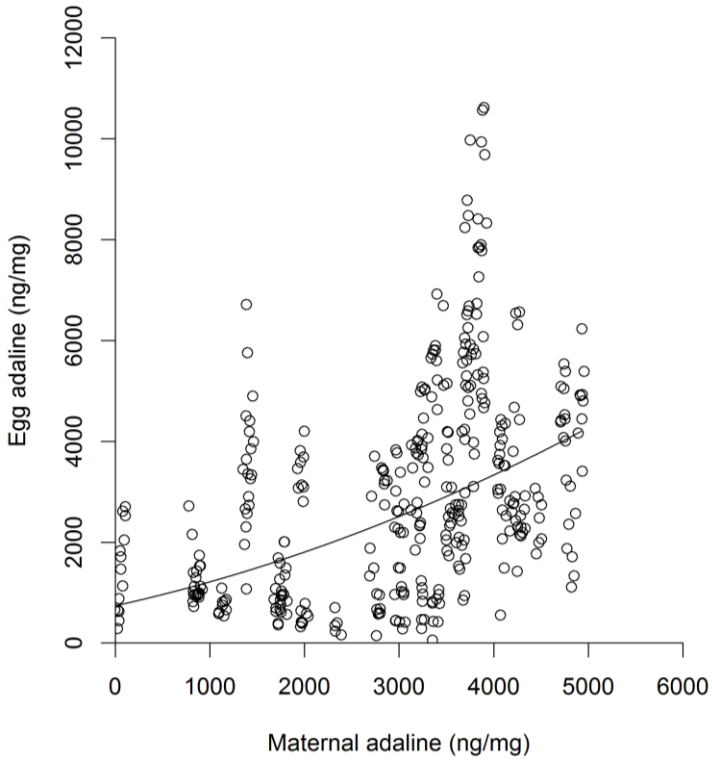

Figure 6. Maternal adaline concentration and the concentration of eggs that they laid. Trend lines are glmm model predictions (see Methods).

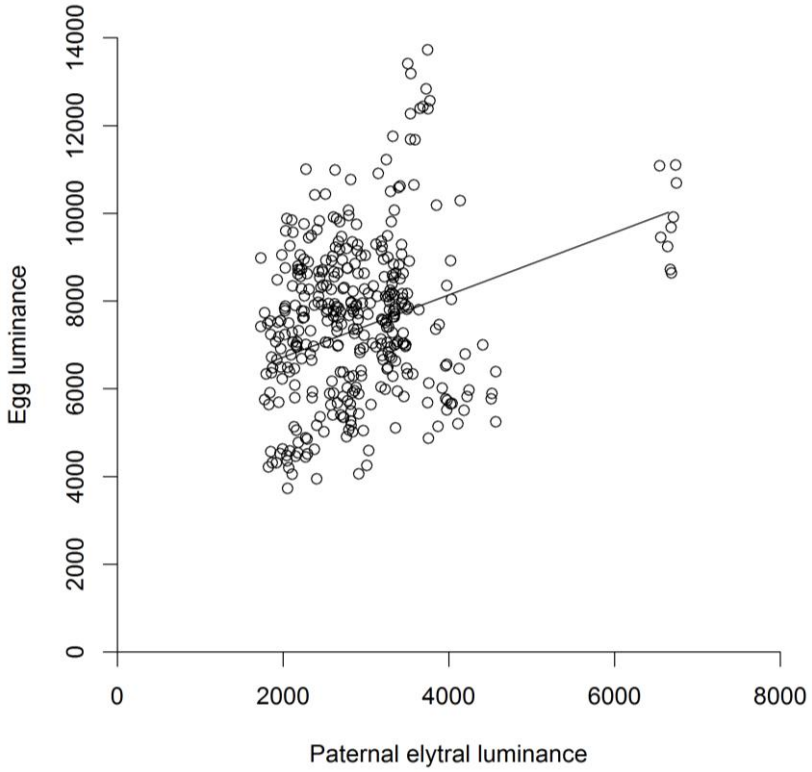

Figure 7. Paternal elytral luminance and egg luminance. Results unchanged when outlier removed.Trend lines are glmm model predictions (see Methods).

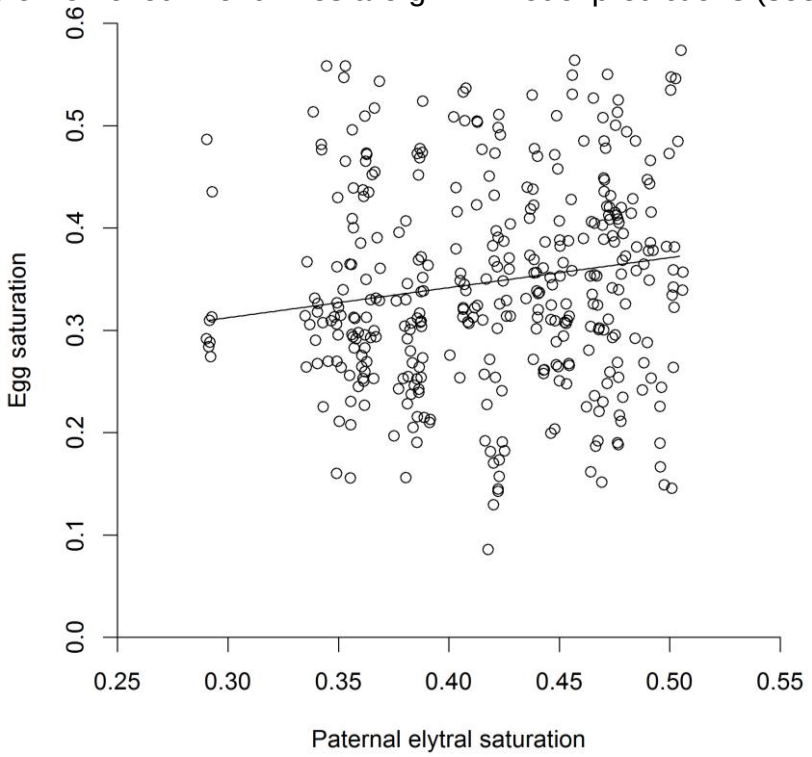

Figure 8. Relationship between paternal elytral saturation and egg saturation. Trend lines are glmm model predictions (see Methods).

Discussion

The risk of offspring predation by conspecifics elicited a stronger and directionally different response, in terms of offspring egg phenotype, to the risk of heterospecific predation. We predicted that in the presence of conspecific larval tracks egg toxin level would decrease and egg coloration would change in order to decrease egg cannibalism risk. There was a decrease in egg adaline content over the three days of the experiment that appeared to be driven by the conspecific predator treatment. This decrease was accompanied, in the conspecific treatment, by a corresponding decrease in egg luminance (lightness). Conversely and also in agreement with predictions, in the heterospecific predator treatment, egg saturation increased over the three days of the experiment, but there was no change in egg toxin level. Furthermore, we predicted that egg toxin level and egg coloration measures would positively correlate with both maternal toxin level and elytral coloration (Winters *et al.* 2014), and paternal toxin level (Camarano *et al.* 2009) and elytral coloration. However only maternal adaline levels predicted egg adaline levels and paternal luminance and saturation predicted egg luminance and saturation, respectively. Together these data provide the first record of a maternally mediated change in offspring colour in response to changes in predation risk and demonstrate additional mechanisms via which variation in aposematic traits may arise within populations.

Response to tracks of offspring predators

Conspecific (A. bipunctata) tracks

Conspecific ladybird larvae benefit from the consumption of conspecific eggs, i.e. cannibalism. Not only are conspecific eggs an energy and nutrient rich resource, but the cannibalistic larvae possess the correct biochemical pathways to process conspecific egg defence chemicals and prevent toxicity (Sloggett & Lorenz 2008). It has also been demonstrated that cannibalistic larvae preferentially consume eggs with a higher toxin (adaline) content (Chapter 3) and that they may be able to sequester adaline from consumed eggs (Laurent *et al.* 2002; Kajita *et al.* 2010). It is therefore in the interest of mothers to reduce any signals of egg toxin content, such as aspects of coloration, which may increase the likelihood of egg detection and consumption in the presence of conspecific cannibals. As in previous studies females were initially reluctant to lay in the presence of conspecific tracks and, perhaps as a consequence, laid both fewer eggs and a smaller number of egg clusters in total (Martini *et al.* 2009). Egg luminance also decreased in the presence of conspecific tracks. Luminance is important for detection, particularly under low light conditions (Kelber *et al.* 2003) such as those found on the underside of leaves, where ladybird eggs are laid (Hodek *et al.* 2012). Decreases in the strength of egg luminance may therefore decrease the risk of detection by predatory conspecific larvae.

There was also a change in the relationship between luminance and toxicity under conspecific predation risk. In contrast to the control and heterospecific

treatments, there was no significant correlation between egg luminance and egg toxin level; i.e. luminance no longer honestly signalled toxin level. Classically when discussing signalling honesty in aposematic species it is in terms of a receiver to whom the chemical defence of the signaller is either unpalatable or toxic (Summers *et al.* 2015). This is not applicable for *A. bipunctata* larvae preying on aposematic conspecific eggs, as it is no longer in the signaller's (egg's) benefit for the receiver (conspecific larvae), to have either the eggs appear conspicuous, and therefore attract attention, or to convay any information about their toxin content that might make them appear more palatable. It may therefore be more appropriate to speak of this change in egg colour as deceptive as opposed to dishonest, although it may also just simply be an example of an uninformative signal. Nonetheless it demonstrates that changes in the nature of the aposematic signals of *A. bipunctata* eggs occur in response to early life selective pressures, specifically predation by conspecifics.

A decrease in egg toxin level was also expected in response to conspecific tracks, as increased egg toxicity in *A. bipunctata* has been linked to higher cannibalism risk (Chapter 3). Contrary to predictions, however, though the overall decrease in egg toxin level after day 0 appeared to be driven by the conspecific treatment, no significant effect or significant interactive effect of treatment on egg toxin level was found. There are two possible explanations for the lack of observed effect of conspecific treatment on egg toxin level, both involving heterospecific predation. Firstly, there may still be a risk when laying in an environment where aphids are present, as they are in this experiment, of predation of future offspring by heterospecifics (Seagraves 2009), and it may

therefore not be selectively advantageous for females to decrease toxin level to too great an extent. Secondly, as egg and adult toxin level is tightly correlated (Winters *et al.* 2014), and toxicity as well as colour is important in deterring predation on adults (Marples *et al.* 1989), decreasing egg toxin level in early life may negatively impact adult survival. Females may therefore have to balance reducing predation on offspring during early and later life. Such antagonistic selection pressures between life stages are not uncommon (Schluter *et al.* 1991; Aguirre *et al.* 2014), though they remain unexplored and previously unidentified in aposematic species.

Heterospecific (C. septempunctata) tracks

In response to heterospecific (*C. septempunctata)* larval tracks, there was no change in toxin level, but there was an increase in egg saturation. The relationship between egg saturation and egg toxin level, and for that matter egg luminance and egg toxin level, was also consistently positive across days. The nature of this relationship, i.e. egg signalling honesty (Blount *et al.* 2009), therefore did not alter under increased heterospecific predation risk as females laying eggs of all toxin levels increased the degree of egg saturation. It is not immediately clear, however why females would increase egg saturation as opposed to egg toxin level. Data on the palatability of *A. bipunctata* eggs to *C. septempunctata* is conflicting (Agarwala & Dixon 1992; Sato & Dixon 2004), but *C. septempunctata* will consume *A. bipunctata* eggs if resource constrained (Hemptinne *et al.* 2000b). However, the sublethal effects of egg consumption are greater for *C. septempunctata* than *H. axyridis* (Hemptinne *et al.* 2000a; Ware *et al.* 2009; Katsanis *et al.* 2013) and *C. septempunctata* larvae are also

more sensitive to changes in the toxin level of heterospecific eggs (Kajita *et al.* 2010). Thus increasing egg toxin level would likely benefit *A. bipunctata* egg survival in the presence of *C. septempunctata*.

One possible explanation is that the lack of increase in egg toxin level reflects the constraints of sibling cannibalism on female investment in offspring. Sibling cannibalism is positively influenced by egg toxin level (Chapter 3), but is only adaptive to mothers in low resource environments where it increases the total number of surviving offspring (Pfennig 1997). Resource levels (i.e. aphid numbers) in this experiment were high and therefore females may have risked maladaptively increasing levels of sibling cannibalism in their offspring if they increased egg toxin level. In contrast, increasing egg saturation increases aposematic signal strength (Arenas *et al.* 2014) and therefore deterrence of heterospecific predators, while having no influence on the levels of sibling cannibalism. This assumption may seem counterintuitive considering the assertion made above about conspecific predators being attracted to more conspicuous eggs. However, newly emerged larvae, the instigators of sibling cannibalism, are likely to differ in their sensory systems from the fourth instar larvae used as non-sibling conspecific predators in this experiment, in particular they are likely to have a more rudimentary visual system (Paulus, 1986; Buschbeck 2014). They also differ in their distance from conspecific eggs; siblings will hatch next to them and non-sibling conspecifics may come across them when foraging (Hironori & Katsuhiro 1997; Perry & Roitberg 2005). Chemical as opposed to visual cues may therefore be more important in

determining cannibalism by siblings than cannibalism by older non-sib conspecifics.

It is also not immediately clear why there was a difference between the two predator treatments in the aspects of egg coloration that were maternally altered, i.e. why did both luminance and saturation not decrease in the conspecific treatment and increase in the heterospecific treatment? For aposematic signals the achromatic component (i.e. luminance) has been demonstrated to be less important than the chromatic component (i.e. saturation and hue) in determining conspicuousness (Arenas *et al.* 2014). It therefore follows that egg saturation, as part of the chromatic component of egg coloration, and not egg luminance, would increase under conditions of high heterospecific predation risk; i.e. when a strong and conspicuous aposematic signal benefitted egg survival. Likewise if under conspecific predation risk it was selectively advantageous to maintain key components of the egg's aposematic signal, due to links between egg and adult coloration (Winters *et al.* 2014), this would explain why no change in egg saturation occurred in this treatment.

It is also important to note that understanding the type and abundance of a predator's visual receptors does not provide information about how that predator processes visual information (Kelber *et al.* 2003). For example, the presence and type of colour-opponent channels, which can enhance the conspicuousness of aposematic species, differs markedly between predator species (Chatterjee & Callaway 2003; Solomon & Lennie 2007). This information is not available for ladybird larvae and as such, though palatability tests have demonstrated larval ability to discriminate between eggs of different

toxin levels (Chapter 3), a greater understanding of larval vision is therefore needed to fully understand the potential biological effect of the observed differences in egg coloration observed here.

Maternal and paternal investment in offspring

Contrary to predictions, while maternal toxin level correlated with egg toxin level, no measure of maternal colour correlated with either measure of egg colour. In contrast, though paternal toxin level had no influence on offspring toxin level, both measures of male elytral coloration were positively correlated with the two measures of egg colour. The influence of maternal condition and maternal toxin level on offspring toxin level is well established (Eisner *et al.* 2000; Hanifin & Brodie 2003; Hutchinson *et al.* 2008; Williams *et al.* 2011), including in aposematic species (Stynoski *et al.* 2014; Winters *et al.* 2014). Examples of paternal contribution to offspring toxin level also abound (reviewed in Eisner *et al.* 2008), but the paternal transfer of toxins is neither universal (Newcombe *et al.* 2013) nor easy to conclusively establish (Camarano *et al.* 2009). Therefore, our results suggest that maternal investment plays a bigger role in offspring toxin level than the paternal transfer of toxins. However, we cannot rule out that the latter occurred, e.g. via sperm or nuptial gifts (Eisner *et al.* 2008), without toxin analysis using labelled compounds (Camarano *et al.* 2009).

The lack of a relationship between maternal colour and egg colour was surprising considering results for toxin level. A possible explanation lies in the link between maternal resource availability and signalling honesty. Theory

predicts that when resources are abundant the conspicuousness and toxicity of aposematic species will cease to be positively correlated (Blount *et al.* 2009). Under high resource conditions individuals are able to increase their toxicity to a point where they are able to ward off attackers and therefore benefit from a decrease in their conspicuousness to predators (Leimar *et al.* 1986). The females used in this experiment were reared and maintained on adlib aphids, i.e. high resource availability. This may therefore explain why there was no association between female toxin level and luminance and only a weak relationship between female toxin level and saturation. Consequently investment in offspring chemical defence may have traded-off against the maintenance of maternal toxin level, but there was unlikely to have been a trade-off between compounds contributing to maternal and offspring coloration (e.g. carotenoids (Blount *et al.* 2012; Winters *et al.* 2014)). Furthermore, it is all the more likely considering the substantial per egg toxin investment in *A. bipunctata*; the adaline concentration of eggs was an order of magnitude greater than maternal concentration. Such a trade-off would have meant that the allocation of toxins to offspring was proportional to a female's ability to produce them, resulting in the observed correlation between maternal and egg toxin content.

The question is raised, however, that if there was less competition between mothers and eggs for resources related to coloration then why did mothers not increase egg coloration disproportionately to egg toxin level and increase the strength of the deterrent warning signal, i.e. signal dishonestly? It is probable that this may have been prevented by mechanisms of honesty enforcement e.g.

‘go slow’ sampling by predators which punishes cheaters (Guilford 1994; Speed & Franks 2014). Such mechanisms prevent females from investing in a way where colour no longer becomes representative of egg toxicity, i.e. from producing dishonestly signalling offspring.

The positive correlation between paternal elytral coloration and egg coloration may have resulted from increased maternal investment in eggs, via an increase in pigment (e.g. carotenoid) quantity, in response to the degree of male elytral coloration. An example of positive differential allocation (DA), where females increase investment in response to increasing male quality (Ratikainen & Kokko 2010; Horvathova *et al.* 2012), this phenomenon is well recorded in relation to male sexual signals. Female mallards (*Anas platyrhynchos*), for example, lay larger eggs with higher albumen lysozyme concentration, and have greater reproductive success after mating with more attractive males (Cunningham & Russell 2000; Giraudeau *et al.* 2011; Sheppard *et al.* 2013). Females of several aposematic species have been shown to prefer males based on their conspicuous coloration (Maan & Cummings 2008; Finkbeiner *et al.* 2014) and ladybird females are known to discriminate between males of different coloured morphs (Majerus *et al.* 1982). Male coloration therefore appears to be multifunctional, acting both as a warning signal to deter predators and a signal of male quality to females (Summers *et al.* 1999). This is the first potential example however, that differential allocation may occur in aposematic species in response to male coloration and highlights an area for future investigation. It is also important to point out that, though unlikely, the direct transfer from fathers, of compounds that contribute to offspring coloration in either seminal

fluid or via nuptial gifts, cannot be ruled out. As with the direct paternal transfer of toxins to offspring, further analysis with labelled chemicals or the artificial manipulation of male attractiveness is needed to verify whether such direct paternal effects contributed to offspring coloration (Camarano *et al.* 2009; Kingma *et al.* 2009)

Summary

We demonstrate changes in egg coloration in response to predation risk for the first time, revealing the dynamic role of maternal effects in determining aposematic coloration. In response to conspecific predation risk egg luminance decreased, indicating that egg coloration became less informative of egg toxin level in the presence of cannibals. Saturation and toxicity did not decrease in response to conspecific cues, possibly due to a strong link between offspring and adult aposematic phenotype and the selective advantage of possessing high levels of chemical defence and an associated warning signal in later life. Such antagonism between selection pressures at different life stages is not uncommon, but as the first potential example in an aposematic species it warrants further investigation.

Heterospecific predation risk resulted in females laying eggs with higher saturation levels but not higher toxicity, presumed to be a result of the constraints associated with increasing sibling cannibalism risk. Maternal toxin level influences offspring toxin level, confirming previous work on chemically defended and aposematic animals. We also provide evidence indicating for the first time that aposematic females may alter investment in offspring based on

paternal warning coloration. Further work is needed to establish the proportion of the paternal effects attributable to direct and indirect effects. However these results open up the possibility that warning signals can impact male fitness not just through influencing his survival and mating opportunities, but via their effect on the survival of the offspring he sires.

CHAPTER 6

General Discussion

In this thesis I have demonstrated that multiple aspects of egg phenotype in *A.bipunctata* are altered by mothers in response to changes in offspring predation risk, cannibalism risk, food availability, and possibly mate 'quality'. This is the first documented example of anticipatory maternal effects (AMEs) in an aposematic species. It is also one of the few studies to consider the role of maternal effects in a species' response to anthropogenic environmental change (Barbosa et al, 2015; Suarez-Ulloa et al., 2015), and it is the first to investigate the role of maternal effects in the response of a species to an invasive predator. The initial questions I posed at the beginning of the thesis are outlined below and I will address the extent to which I have answered each question and discuss the implications of my findings in the following chapter.

Chapter 2) Do female *A.bipunctata* modify egg toxin level, in response to the presence of an invasive offspring predator? Is there a trade-off between egg number and egg toxin level?

Chapter 3) How do *A.bipunctata* modify offspring phenotype in response to antagonistic selection pressures?

Chapter 4) How does maternal phenotypic variation, in this case female morph, lead to variation in anticipatory maternal effects; i.e. the extent to which offspring phenotype is modified in response to predator presence?

Chapter 5) How does maternal and paternal phenotypic variation influence offspring phenotype under the risk of predation and the risk of cannibalism?

Modification of egg toxin level in response to offspring predation risk

No change in egg toxin level was observed in response to elevated offspring predation risk, from either heterospecific invasive (*H.axyridis* larvae), heterospecific native (*C.septempunctata* larvae), or unrelated conspecific cannibal (*A.bipunctata* larvae) predators. That is there was no evidence of an anticipatory maternal effect (AME) mediated change in egg toxin level in response to maternal exposure to an increase in offspring predation risk. Previous authors have argued that the overall weak evidence for AMEs stems from researchers looking for them in systems, where there are no reliable cues of the future offspring environment (Uller *et al.* 2013). This is unlikely to be the case here as female laying behaviour and other aspects of offspring phenotype, including cluster size, number and location laid (oviposition site) changed in response to all three types of predation risk (Chapters 2 -5), confirming results from previous studies (Chapter 2 [Table 1]; Agarwala & Dixon 1993; Kajita *et al.* 2006).

In Chapter 2 an argument put forward to explain the lack of increasing egg toxin level in the face of *H.axyridis* predation, is that females were physiologically constrained from producing more toxic eggs when laying large clusters. An alternative explanation may be that increasing egg number and/or cluster size (Chapters 2 & 3) is a more adaptive response than increasing egg toxin level in

the presence of *H.axyridis* larvae. The latter have a high tolerance of heterospecific toxins (Pell *et al.* 2008), which may mean that changes in egg toxin level would not affect overall predation rates. In contrast, prey aggregations (i.e. egg clusters), and overall prey number (i.e. egg number) are known to increase offspring survival even when some prey are palatable, through both increased aposematic signal strength and the dilution effect (Chapter 1; Turner & Pitcher 1986; Rowland *et al.* 2010). However, if this were the case then changes in egg toxin level in response to predators with lower toxin tolerances, e.g. *C.septempunctata* larvae (Agarwala & Dixon 1992), would have been expected, but these were not seen (Chapter 5).

There may also have been costs associated with the alteration of egg toxin level that were not outweighed by the benefits accrued through decreased predation. Such costs could be physiological (Zera & Harshman 2001) or associated with the concurrent increased risk of sibling cannibalism that accompanies increases in egg toxin level (Chapter 3). In Chapter 3 I demonstrate that increased egg toxin levels raise the risk of conspecific cannibalism and that females increase egg toxin level in conditions when sibling cannibalism is beneficial to maternal fitness; i.e. resource availability is low. However, in conditions of high resource availability, as is the case for all of my tests of predation risk effects, sibling cannibalism is not beneficial. Therefore there is a risk that increasing egg toxin levels under such conditions, though it may deter predators, might also increase levels of sibling cannibalism in a way that would be maladaptive to maternal fitness (Pfennig 1997). Thus maternal response to egg predators may be constrained somewhat by sibling cannibalism risk.

It is also worth noting that the animals used in all of the studies were from cultures, either from Bioline Syngenta or Gardening Naturally (UK), not wild-collected. The use of cultured ladybirds when studying their toxic properties is a common practice (e.g. Vilcinskas *et al.* 2013) and in the case of *A.bipunctata* was necessary due to their recent decline in the wild and particularly low numbers during 2012-2014 owing to adverse climatic conditions (Comont *et al.* 2012; Roy *et al.* 2012; Comont *et al.* 2014). Furthermore, comparison of the toxin level of eggs laid by a small number of wild *A.bipunctata* and those from the cultured individuals used in this thesis revealed no significant difference (Appendix V).

Trade-offs between egg toxin level and egg number

Trade-offs between offspring number and per-offspring maternal investment are predicted by theory (Smith & Fretwell 1974; Parker & Begon 1986; Bernardo 1996b; Olofsson *et al.* 2009; Rees *et al.* 2010) and have been identified in a number of taxa (Deas & Hunter 2012; Riesch *et al.* 2012). Empirical support is, however, equivocal (Plaistow *et al.* 2007; Monteith *et al.* 2012). In treatments with *H.axyridis* tracks, an increase in either cluster size or number corresponded to a decrease or lack of change in egg toxin level in *A.bipunctata* (Chapters 2 & 3). However, no such trade-off was observed between egg toxin level and egg number across all other treatments in *A.bipunctata* (Chapters 2-5). There was also a positive correlation between egg number and cluster size and egg toxin level in treatments not involving *H.axyridis*, which in turn

correlated with maternal size and toxin level, respectively. The relationship between maternal size and both fecundity and offspring size is well established (Berkeley *et al.* 2004; Steiger 2013; Saenz-Agudelo *et al.* 2015). In ladybirds the number and size of ovarioles is known to scale positively with female size (Dixon & Guo 1993; Ware *et al.* 2008), and female toxin level also scales positively with size (Chapters 4 & 5). Larger females therefore have both greater toxin levels and ability to produce eggs; they consequently produce a greater number of more toxic eggs than smaller females.

Offspring phenotype, e.g. size, is thought to reside at an optimum for maternal, as opposed to offspring, fitness (Lack 1947b; Smith & Fretwell 1974; Janzen & Warner 2009). This optimal phenotype varies depending on the environment (Parker & Begon 1986), and differing environmental conditions in both space and time can therefore reveal the presence of trade-offs (Fox *et al.* 1997b; Nakajima & Fujisaki 2012; Rollinson & Hutchings 2013). It is arguably unsurprising, therefore, that the only time a possible trade-off between egg toxin level and egg number was observed was when; egg toxin level increased in the absence of aphids but only when predators were also absent as egg number had increased in response to predator presence (Chapter 3). That is, only once did the environment vary in a way that required a change in the aspect of offspring phenotype that we were measuring. It is also worth noting that as the majority of the experimental work carried out was predominantly concerned with AMEs, they only provided a snapshot of the full ladybird reproductive period (oviposition period). Unlike salmon, on which some of the seminal studies on reproductive trade-offs are carried out (Smith & Fretwell 1974; Rollinson &

Hutchings 2013), ladybirds have multiple reproductive events (iteroparity). As such, although trade-offs in some iteroparous insects have been identified at individual laying events (Fox *et al.* 1997a), in a large number of cases they become evident only when looking at either reproductive rate or total life-time fecundity, i.e. when looking at a larger window of a females' reproductive life (e.g. Guisande & Gliwicz 1992; Vijendravarma *et al.* 2010). Consequently further work looking at the long term influence of variation in offspring resource availability on maternal investment is needed, to elucidate the extent of the potential trade-off between egg number and egg toxin level in *A.bipunctata*.

Predation risk, egg colour and signal honesty

In contrast to egg toxin level, aspects of egg coloration were altered after maternal exposure to offspring predator cues, although the nature of this change differed according to the species of predator (Figure 1). When cues of heterospecific predators, to whom aposematic defences may deter egg predation, were present, markers of egg coloration that are linked to conspicuousness increased (Chapters 4 & 5). In the presence of *H.axyridis* larval tracks, egg luminance increased (Chapter 4) and in the presence of *C.septempunctata* larval tracks, egg saturation increased (Chapter 5). In contrast, in the presence of unrelated conspecific predators (cannibals), egg luminance decreased (Chapter 5).

The decrease in egg luminance in the presence of conspecific larval tracks may be more appropriately referred to as deceptive as opposed to dishonest, as signal honesty in aposematic species is framed in the context of a signaller who is either unpalatable or toxic to the a receiver (Summers *et al.* 2015). Though

A.bipunctata eggs are aposematic, when the risk of conspecific cannibalism is high, it is no longer in the signaller's (egg's) benefit for the receiver (conspecific larvae) to have either the eggs appear conspicuous, and therefore have their attention drawn to them, or to convey any information about their toxin content which might make them appear more palatable. It could, however, be equally as valid to argue that this is simply a case of uninformative signalling. Nonetheless, irrespective of explanation, it demonstrates that changes in the nature of the aposematic signals of *A.bipunctata* eggs occur in response to early life selective pressures, specifically predation by conspecifics.

Why the three predator treatments differed in maternally altered aspects of egg coloration is unclear; i.e. why did not both luminance and saturation increase in the presence of tracks of each of the heterospecific larvae and decrease in the presence of conspecific larval tracks (Chapters 4 & 5)? In chapter 5 I argue that different aspects of egg coloration are altered in response to heterospecific as opposed to conspecific tracks, due to the differing importance of saturation and luminance in the determination of aposematic signals. Saturation is key in determining the conspicuousness of an aposematic species (Arenas et al., 2014), whereas luminance plays a greater role in detection under low light conditions (Kelber *et al.* 2003), such as the underside of leaves where ladybird eggs are laid (Hodek *et al.* 2012). Consequently it is plausible that egg saturation increased in the presence of heterospecific predator (*C.septempunctata*) tracks in order to strengthen the deterrent aposematic signal. Egg saturation was maintained, as opposed to reduced, when the risk of conspecific predation was high, most likely because of the strong links between

egg and adult coloration (Winters et al. 2014), and between adult conspicuousness and survival (Arenas et al. 2015). Following this logic an increase in egg saturation as opposed to luminance may have also been expected in response to cues of the heterospecific predator, *H.axyridis*, however this was not found. Egg luminance, not egg saturation, was increased in the presence of *H.axyridis* larvae, and it is not yet clear why such a difference in components of egg coloration should have occurred in the response to the two heterospecific larval predators.

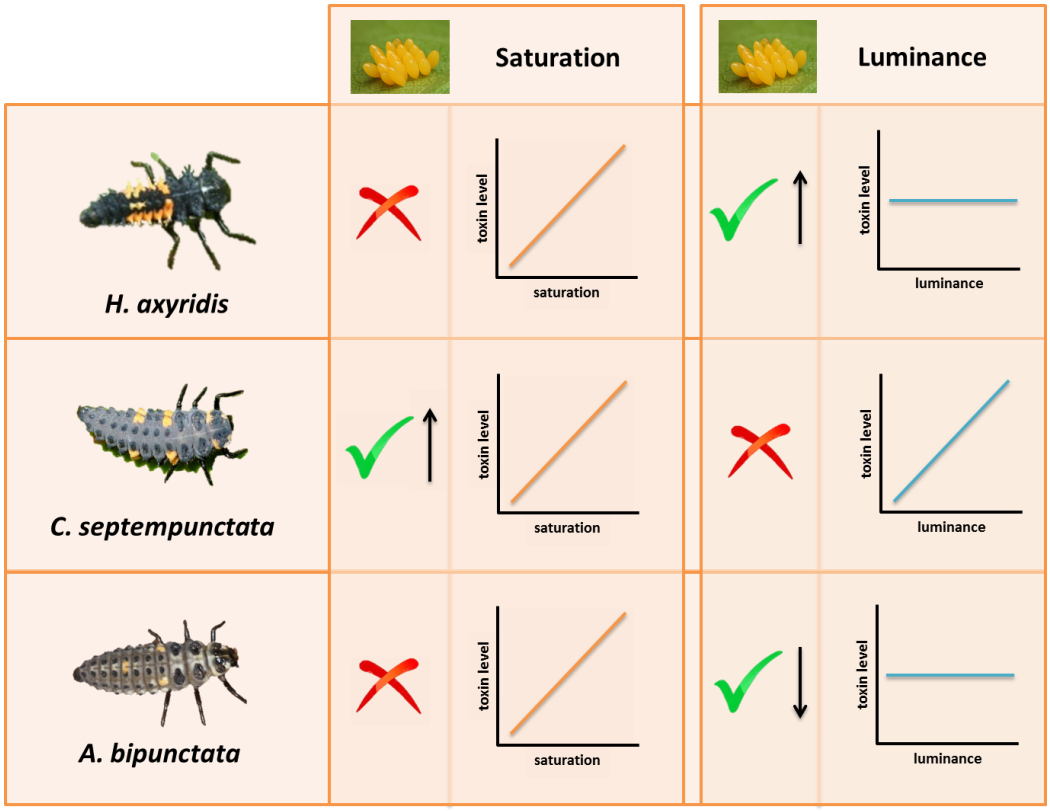

Figure 1. Infographic illustrating the effect of reliable cues of larval ladybird egg predators, illustrated on the left, on the consequent color (saturation and luminance) of eggs laid by *A.bipunctata* females (red cross for no effect and green tick for an effect, with an arrow to indicate direction) and the relationship between each measure of coloration and egg toxin level (plots).

Despite the complex pattern of changes in egg colour observed in response to predation risk, the relationship between egg saturation and toxin level remained consistently positive across all three predator treatments, i.e. egg saturation honestly signaled toxin content (Figure 1; Chapters 4 & 5). Such signal honesty has been detected in a number of aposematic organisms (Vidal-Codero et al. 2012; Bezzerides et al.2007; Mann & Cummings 2012) and in line with a previous study on eggs of a different ladybird species, though saturation positively correlated with toxin content, luminance did not always show the same relationship (Winters et al. 2014). This may be because, due to its greater role in the determination of warning coloration, mechanisms of honesty enforcement have a stronger effect on the relationship between saturation and toxin level than luminance and toxin level, in aposematic species.

Two non-mutually exclusive mechanisms for the maintenance of signal honesty in aposematic species are the ‘resource allocation’ and ‘go slow’ hypotheses (Holen & Svennungsen 2012; Summers *et al.* 2015). Both mechanisms prevent females from investing in such a way that colour is no longer representative of egg toxicity. Each involves a different form of ‘handicap’, that is a cost associated with the signal which maintains its honesty (Zahavi, 1975, 1977). In the case of the resource allocation hypothesis this handicap is physiological (Blount et al, 2009). The production of both signals and defence chemicals is energetically costly (Holloway et al., 1991), and not only that but those antioxidant pigments involved in the production of conspicuous signals (e.g. carotenoids) may also be important in preventing autotoxicity when producing and storing toxins, resulting in physiologically mediated competition for a shared

resource (Blount et al, 2009). Following this, the ‘resource allocation’ hypothesis predicts a positive correlation between toxin level and conspicuousness within species and populations when resources are limiting, as observed in paper wasps (Manuel Vidal-Cordero *et al.* 2012), ladybird adults (Bezzerides *et al.* 2007; Arenas *et al.* 2015b), and in two-spot and seven-spot ladybird eggs (Chapters 3 & 4; Winters *et al.* 2014a). However the only study to explicitly test the hypothesis provided a complex picture of conflicting results (Blount *et al.* 2012). Using the seven-spot ladybird (*Coccinella septempunctata*) Blount et al. (2012) demonstrated that resource limitation reduced carotenoid and precoccinelline (a precursor to the toxin coccinelline) levels in both sexes. Authors also showed that a positive correlation between carotenoid and coccinelline levels existed in females across treatments, but that a negative correlation occurred between the two, in males.

The ‘go slow’ hypothesis puts the cost of conspicuousness in terms of predator detection, where more conspicuous prey are more likely to be detected and therefore attacked by naïve predators (Guilford & Dawkins 1993), learning via ‘trial and error’ being a key component of the avoidance of aposematic organisms by many predators (Skelhorn *et al.* 2008). The striking coloration will cause predators to ‘go slow’ when handling aposematic prey (Guilford 1994), but only toxic prey will be able to deter such predators from eating them, the cost of dishonesty (i.e. falsely signalling high toxicity) is therefore consumption by predators. In terms of variation in the degree of conspicuousness, less conspicuous and less toxic (honest) prey will therefore be less detectable than more conspicuous (honest) aposematic prey and so will have a lower detection

and attack rate but will still signal their unpalatability to a greater extent than palatable prey. Work using bird predators has demonstrated that naïve predators do indeed attack aposematically coloured prey less readily than non-aposematic coloured prey and this bias is reinforced through bitter tasting compounds (Gamberale-Stille & Guilford 2004; Skelhorn & Rowe 2006; Rowland *et al.* 2013). As stated above both the 'resource allocation' and 'go slow' hypotheses are non-mutually exclusive (Holen & Svennungsen 2012; Summers *et al.* 2015) and therefore further work, for example predation tests, would be required to fully establish the role of each in maintaining honesty in the eggs of *A. bipunctata*.

Response to antagonistic selection pressures

In chapter 3 we demonstrate the importance studying maternal effects, especially AMEs, not just in response to single environmental variables, but in the context of the interactive and potentially antagonistic factors that affect offspring survival and fitness (Kuijper *et al.* 2014). This chapter deals with the maternal response to two environmental variables, predation and resource availability, that positively covary in the wild, and high levels of which favour different forms of the same offspring trait, high and low egg toxicity respectively. Predators are deterred and cannibals attracted by egg toxins (Chapter 3; Kajita *et al.* 2010) and cannibalism is not selectively advantageous for mothers under resource abundant conditions (Pfennig 1997; Perry & Roitberg 2005b). We found that *A. bipunctata* females laid eggs with a higher toxin content in the absence of aphids, but only when predator cues were also absent. A palatability test showed that conspecific larvae preferentially consumed eggs of a higher

toxin level, suggesting that females may increase toxin level to increase sibling cannibalism in the absence of aphids, as cannibalism benefits maternal fitness in low resource environments. This response was likely constrained when predator cues were also present due to the larger number of eggs laid in the presence of predator cues resulting in a trade-off between egg number and egg alkaloid level.

These results differ from work on opposing selection pressures on intra-generational rather than trans-generational plasticity in the wood frog (*Rana sylvatica*), which result in phenotypes displaying a balance between the two phenotypic optima (Relyea 2004; Relyea & Auld 2005). However, they highlight the importance of studying maternal effects and other modes of transgenerational inheritance in the context of the multiple selective pressures that exist in an organism's environment (Kuijper *et al.* 2014). Moreover the idea that AMEs do not occur due to the constraints imposed by other abiotic or biotic variables, may further help to explain why the overall evidence for AMEs in systems is weak even when reliable cues of the future offspring environment exist (Uller *et al.* 2013).

Parental phenotypic variation

Maternal

The eggs of melanic *A.bipunctata* mothers, although colourfully aposematic like the eggs of red (typica) *A.bipunctata* mothers, had lower toxin content and values of saturation and luminance (Chapter 4). One explanation given for this difference, though speculative, rests on the basis that there is a trade-off between maternal survival and reproductive investment (Trivers, 1974; Stearns,

1992). This trade-off and the costs associated with melanism (predatory and physiological; Hegna *et al.* 2013; Roulin et al. 2016) predict that melanic individuals would make a smaller per offspring investment in terms of both toxins and the pigments associated with coloration e.g. carotenoids. If there was such a trade-off between maternal and offspring levels of chemical defence and coloration, reproductive investment would be expected to have been proportional to maternal levels of specific 'resources' e.g. toxins and carotenoids. Accordingly, I found a positive correlation between maternal mass and egg number (Chapter 3 & 5) and between maternal toxin concentration and egg toxin concentration (Chapter 5), though these trends were not consistent across all experiments (Chapters 2 & 3). In chapter 3 though maternal toxin level did not correlate directly with egg adaline level, older females laid eggs with a higher toxin content and further work demonstrated that adult toxin level increased with age (Appendix IV). No relationship was found between either maternal elytral saturation or luminance and egg saturation or luminance, respectively (Chapter 5). This is surprising considering the relationship between female and egg toxin levels. It suggests that elytral coloration, which is determined during adult eclosion (Blount *et al.* 2012), may not have been representative of circulating levels of carotenoids at the time of the experiment (approx. 20 days post eclosion). This is all the more likely considering that females were reared on an *ad lib.* supply of aphids and that carotenoids are obtained from the diet as opposed to being endogenously produced (Goodwin, 1984).

Though not investigated here maternal state or ‘condition’ (Hill, 2011) can also influence maternal investment and therefore maternal effects (Badyaev & Uller 2009; Wolf & Wade 2009). For example, in many iteroparous insects egg laying behaviour, including site choice and egg phenotype, is determined by a trade-off between egg and time limitation (Rosenheim 1999). Females are time limited when they have a greater number of eggs than time or sites on which to lay these eggs, and they are egg limited when the reverse is true (Rosenheim 1996; Sevenster *et al.* 1998). Egg limited females are more discriminatory than time limited females about the quality of the sites where they lay their eggs, that is they are less likely to lay in the presence of predators or competitors (e.g. Xu *et al.* 2012). However, though less discriminatory in where they lay their eggs, time limited females have been shown to invest more in egg protection when laying in risky sites, than egg limited females (Deas & Hunter 2014), an example of the interaction between different forms of maternal effect.

The females used in our experiments were all of a similar age (mated at 15-25 days) and had not yet reached the age of peak fecundity, however they were mated a considerable time after reaching sexual maturity (6-10 days) (Hodek *et al.* 2012). They were therefore most likely to have a had high egg load and to have been time- as opposed to egg limited. In ladybirds older females are more likely to lay eggs in the presence of predator tracks than younger females (Frechette *et al.* 2004). However whether differences in egg load also alter maternal investment in ladybirds is unknown and it would be interesting to investigate these concepts further and assess the impact that they have on investment in aposematic species.

Paternal

The positive correlation between paternal elytral coloration and the coloration of the eggs that they sired, may have resulted from the alteration of female reproductive investment in response to variation in male coloration. Female investment in eggs may have increased, via an increase in pigment (e.g. carotenoid) quantity, in response to the degree of male elytral coloration (positive 'differential allocation' (DA); Ratikainen & Kokko 2010; Horvathova *et al.* 2012). Females of several aposematic species have been shown to prefer males based on their conspicuous coloration (Maan & Cummings 2008; Finkbeiner *et al.* 2014) and ladybird females are known to discriminate between males of different coloured morphs (Majerus *et al.* 1982a), but this is the first indication of differential allocation in an aposematic species. It is also important to point out that though unlikely the contribution of the direct transfer of compounds that determine offspring coloration, via either seminal fluid or nuptial gifts (Eisner *et al.* 2008), cannot be ruled out. Though the explanations are not mutually exclusive, this is the first indication of paternal effects in an aposematic species, and further analysis with labelled chemicals or the artificial manipulation of male attractiveness is needed to ascertain which mechanisms underlie the observed differences (Camarano *et al.* 2009; Kingma *et al.* 2009).

Variance in offspring phenotype as opposed to mean phenotype

The focus throughout this thesis and in much of the literature on maternal effects is on changes in the mean of particular offspring phenotypic traits e.g. mean egg size or mean egg toxin level. Such changes are anticipated to occur in response to predictable variation in the offspring environment (Marshall &

Uller 2007), whereas when environments are unpredictably variable females can increase their fitness by increasing variation in offspring phenotype ('diversified bet-hedging'; Philippi & Seger 1989; Olofsson *et al.* 2009). This increase in variability boosts maternal fitness by decreasing the variability in offspring fitness between reproductive bouts and between individuals using the same strategy (of the same genotype); that is fitness variance is reduced and an individual's geometric mean fitness is increased (Philippi & Seger 1989; Starrfelt & Kokko 2012). Such bet-hedging was previously considered as a fixed strategy to deal with unpredictable environments (Seger & Brockmann 1987), however it is clear that as well as varying predictably or unpredictably, environments vary in the degree of their unpredictability (Morrongiello *et al.* 2012). Under such scenarios a strategy of dynamic bet-hedging, whereby females can adjust the variability in the phenotypes of a particular cohort (e.g. cluster) of offspring, may be selectively advantageous (Crean & Marshall 2009). Dynamic bet-hedging is predicted to evolve only under a specific set of conditions where environmental variation falls within a narrow range and cues of the future environment are absent (Proulx and Teotonio, 2015). In the handful of empirical studies investigating the phenomenon empirical support is weak (Dey *et al.* 2016; Walsh et al. 2016), however its importance for aposematic species has yet to be investigated. This is surprising considering the large variation in toxicity and both warning signal strength and honesty within and between populations and species (Speed *et al.* 2012; Summers *et al.* 2015). The simple existence of variability in a phenotypic trait could be an artefact of physiological constraint rather than a bet-hedging strategy per se (e.g. as with

egg size; Beaumont et al 2009; Fox and Czesak, 2000). However, variation in toxin defence within a group or population of aposematic species has proven fitness benefits, decreasing predation by increasing the unpredictability of toxin load upon predators (Barnett *et al.* 2014). Therefore though dynamic-bet hedging is unlikely to have been employed in the system utilised in this thesis, due to the predictability of environmental variation and the high repeatability of egg phenotype measurements (i.e. within cohort/cluster variation was low), its role in other aposematic systems merits further investigation.

Biological and ecological relevance of results

As stated above, changes in egg toxin level in response to maternal exposure to cues of reduced resource availability (Chapter 3), and the alteration of egg coloration in response to offspring cannibal and predator cues (Chapters 4 & 5), were both identified in *A.bipunctata*. When discussing the ecological relevance of these results it is important to consider the analytical methods used to assess them. The quantification of toxin levels in chemically defended species via chemical analysis is a commonly used analytical method in determining differences both within and between species in toxicity (Speed *et al*. 2012). Yet, just as it is important to measure visual signals in the context of the animals that are receiving them (Endler 1978; Endler & Mielke 2005; Stevens *et al.* 2007) so the ecological relevancy of quantitative variation in prey toxin level, i.e. differences in palatability or toxicity, can arguably only be unequivocally evaluated via the observation of feeding behaviour and the physiological effects of consumed toxins on predators (Skelhorn 2016).

Predation and cannibalism risk

We demonstrate that cannibals are sensitive to the quantitative variation in conspecific egg toxin content (Chapter 3). However, we do not know whether the changes in egg coloration and distribution (clustering) identified in the presence of heterospecific larval tracks will actually decrease egg heterospecific predation risk. Theory and empirical work in other systems suggests that both should increase predator aversion (Guilford 1988; Gamberale & Tullberg 1996, 1998; Finkbeiner *et al.* 2012; Rowland *et al.* 2013; Arenas *et al.* 2015b), however there is considerable variation between predator species in their predation of aposematic prey (Nokelainen *et al.* 2014). This is certainly true of ladybird larvae, which vary considerably among species in their tendency to attack and consume the eggs of other ladybird species (Appendix II). *H.axyridis* larvae are more voracious than *C.septempunctata* larvae (Agarwala & Dixon 1992; Katsanis *et al.* 2013) and consequently any deterrent effects caused by the changes in *A.bipunctata* egg coloration or clustering are likely to be more pronounced in the latter opposed to the former predatory species. Further experimental work is evidently needed in order to verify the effects of egg coloration on predation by *H.axyridis* larvae. However, the literature on *H.axyridis* voracity suggests that such maternally-mediated changes in egg phenotype are unlikely to significantly alter the asymmetrical intraguild predation relationship between *H.axyridis* and *A.bipunctata* (Kajita *et al.* 2006; Ware & Majerus 2008; Katsanis *et al.* 2013).

Predation rates of aposematic prey also vary considerably not just between predator species, but also with the population (Exnerova *et al.* 2015) or ‘breed’

(H.Rowlands, *personal communication*) of predator. This is pertinent when considering invasive predators such as *H.axyridis*, where large differences in phenotype between individuals on and behind the invasion front have been recorded (Phillips *et al.* 2006). Future studies could assess whether such differences in predatory behaviour exist between invasive populations of *H.axyridis* at different distances from the invasion front, and also whether these differences influence their predatory response to different colours and cluster sizes of *A.bipunctata* eggs. Population-based differences are also relevant to ladybird egg cannibalism, as the prevalence of cannibalistic larval behaviour is known to vary between populations of ladybirds (Tayeh *et al.* 2014). It would therefore also be interesting to investigate the role of larval response to egg toxin level in this variation, i.e. do larvae vary between populations in their degree of preference for conspecific eggs with high toxin content?

Additionally, predator experience (Skelhorn & Rowe 2007a; Mappes *et al.* 2014), state (Barnett *et al.* 2007) and even developmental history (Bloxham *et al.* 2014) can also influence predator behaviour. State-dependent models (Kokko *et al.* 2003; Sherratt 2003) and their empirical tests (Barnett *et al.* 2007; Skelhorn & Rowe 2007b; Barnett *et al.* 2012; Bloxham *et al.* 2014) have shown that predators are more likely to eat aposematic prey if their current nutritional state is low (i.e. they are hungry) or if they had a poor developmental diet. In ladybird larvae heterospecific predation and cannibalism is inversely related to the abundance of other food sources (Agarwala & Dixon 1992; Rondoni *et al.* 2012), though whether this is due to larval nutritional state or increased encounter rate with heterospecific prey is unknown. *Harmonia axyridis* larvae

are also more likely to eat heterospecific eggs if they had experienced a poor diet during early development (Ingels *et al.* 2015). This change in predator behaviour does not occur because predators are less discriminatory; they make state dependent decisions based on the trade-off between their current nutritional status and toxin load (Barnett *et al.* 2007; Skelhorn & Rowe 2007b). For example the European starling (*Sturnus vulgaris*) is able to discriminate between mildly and moderately defended prey using a colour cue, and preferentially consumes mildly over moderately defended prey even when in a low nutritional state (Barnett *et al.* 2012). Ladybird larvae are able to distinguish between eggs of different species and toxin content (Kajita *et al.* 2010) and it therefore seems reasonable to predict that any effect that changes in colour and cluster size of *A.bipunctata* eggs had on predation by heterospecific larvae, would not differ with larval nutritional state. The preference of cannibalistic larvae for high-toxin eggs would be also be expected to remain or perhaps even strengthen when larval energy stores are low, as the energetic benefit of eating conspecific eggs with a higher toxin content is greater for cannibals (Kajita *et al.* 2010).

Maternally-mediated changes in egg coloration were measured in the context of the visual system of the predators to which the females were responding, i.e. ladybird larvae (Chapters 4 & 5).However, understanding the type and abundance of a predator's visual receptors does not provide information about how that predator processes visual information (Kelber *et al.* 2003). For example, the presence and type of colour-opponent channels, which can enhance the conspicuousness of aposematic species, differs markedly between

predator species (Chatterjee & Callaway 2003; Solomon & Lennie 2007). This information is not available for ladybird larvae meaning that we are unable to state conclusively whether the recorded changes in egg luminance and saturation influence egg detection by the larvae. Consequently although palatability tests have demonstrated larval ability to discriminate between eggs of different toxin levels (Chapter 3; Kajita *et al.* 2010), a greater understanding of larval vision is therefore needed to fully understand the potential biological effect of the differences in egg coloration observed here.

Furthermore, like many beetles, ladybirds are highly chemically motivated (Hodek *et al.* 2012). In some ladybird species adult individuals produce volatile chemicals in conjunction with warning coloration to advertise their toxicity (Guilford *et al .*1987; Marples *et al.*, 1994). Chemical aposematism has not received the same level of attention in evolutionary ecology as aposematism that utilises visual signals, and is a fertile area for future research, for example far less is understood about its role in mimicry or intraspecific signalling honesty (Rowe & Guilford 1999; Weldon 2013). In the ladybird *Hippodamia convergens,* warning odour production in adults correlates positively with both warning coloration and toxin level (Wheeler et al. 2015). In ladybird eggs cuticular hydrocarbons (CHCs) on the egg surface are also known to play a key role in the distinction between conspecific and heterospecific eggs by predatory ladybird larvae (Hemptinne et al. 2000b). Such chemicals could act as signals of the egg's toxin content and therefore influence its palatability (Skelhorn & Rowe 2009, 2010), potentially reinforcing signals of warning coloration (Rowland et al. 2010; Skelhorn 2011). It is therefore important to acknowledge

that colour may not be the only way that ladybird eggs may communicate their toxin level to larval predators. Further work is needed to investigate this idea and also to test whether possible chemical signals of egg toxicity are maternally altered in response to offspring environmental cues in a way that mirrors changes in the colour component of the multimodal aposematic signal; i.e. increased in response to heterospecific larvae and decreased in response to cannibals.

Developmental effects of predation and cannibalism

We also did not address the effects of cannibalism or heterospecific predation on larval phenotype and overall fitness. Cannibalism has positive effects on the fitness of the cannibals (Pfennig 1997) and ladybirds are no exception (Ware *et al.* 2008; Ware *et al.* 2009). Conspecific egg consumption by larvae has both positive nutritional and defence benefits (Kajita *et al.* 2010) as cannibals have the correct metabolic pathways to process conspecific chemicals (Sloggett & Davis 2010). In contrast due to interspecific variation in egg toxicity, the effect of eating heterospecific eggs depends on both the species of predator and prey, with consumption leading to either positive (increasing mass and survival) or negative (retardation of development, lower body mass and increased mortality) effects on larvae (Hemptinne *et al.* 2000a; Ware *et al.* 2009). In addition to this asymmetry larvae are also affected by intraspecific variation in heterospecific egg toxin level, with more toxic eggs having a greater negative effect on larval growth and survival (Kajita *et al.* 2010). Therefore despite the nutritional benefit the consumption of heterospecific eggs during development may contribute to a degree of physiological stress due to the toxin load of the eggs (Ware *et al*.

2009). Stress during early life can have consequences for many aspects of adult phenotype, including size, physiology and behaviour (Monaghan 2008). Even relatively short periods of early life food stress can influence cognitive ability (Nettle *et al.* 2015) and foraging strategy (Andrews *et al.* 2015; Bateson *et al.* 2015), including predation of aposematic species (Bloxham *et al.* 2014). In ladybirds, sustained low food availability or quality during early life, affects adult phenotype and fitness (Agarwala *et al.* 2008; Ware *et al.* 2008; Blount *et al.* 2012). However to date there has been no investigation into the effect of either cannibalism or heterospecific predation on adult warning coloration, despite the important link between early life resource availability and both adult warning coloration and toxin content (Blount *et al.* 2012).

Summary

In summary *A.bipunctata* mothers do not alter egg toxin level in response to predation risk from heterospecific ladybird larvae, but they do manipulate sibling cannibalism levels in offspring through the alteration of egg toxin level in a resource availability dependent manner. Our results suggest that changes in toxin level in response to egg predation risk from heterospecific predators is constrained under resource abundant conditions, by the influence of egg toxin level on sibling cannibalism. Egg colour is altered in response to predation risk, in a predator-specific way. Egg signal honesty is maintained in the presence of heterospecific predators, but deception is employed in face of conspecific cannibals. Independent of the effects of offspring environment, egg toxin level

and colour are also influenced by maternal phenotype (both morph and toxin level) and paternal phenotype (elytral coloration).

This study provides the first comprehensive investigation into the role of maternal effects in mediating the response of a species to anthropogenically driven environmental change. The results indicate that it is unlikely that maternally mediated changes in egg phenotype will improve the survival of *A.bipunctata* offspring in the face of predation from the invasive larval predator *H.axyridis*. They do, however, demonstrate the importance of studying maternal effects in the context of the multiple environmental factors which more accurately represent the complex environments in which organisms live and evolve, corroborating recent theoretical predictions. Finally I provide evidence of the multifaceted nature of parental effects in aposematic species and reveal the role that they may play in shaping the variation in defence and warning coloration observed in adult populations.

Appendix I

Table summarising the extent to which the eggs of different ladybird species differ in their palatability and toxicity to predatory ladybird larvae, both conspecific and heterospecific.

Species consuming eggs	**Life stage consuming eggs**	**Species of eggs consumed**	**Palatibility**		**Toxicity**				**Reference**
			Unpalatibile	Palatible	Positive effect	None	Sublethal	Lethal	
Adalia bipunctata	Adult	*Adalia decempunctata*	**x**						**Agarwala and Dixon (1992)**
		Ceratomegilla undecimnotata	**x**						**Agarwala and Dixon (1992)**
		Coccinella septempunctata	**x**						**Agarwala and Dixon (1992)**
		Harmonia axyridis	**x**						**Burgio et al (2002)**
	Larvae	*Adalia decempunctata*	**x**						**Agarwala and Dixon (1992)**
		Ceratomegilla	**x**						**Agarwala and Dixon**

		undecimnotata							**(1992)**
Adalia bipunctata	Larvae	*Coccinella septempunctata*	**x**						**Hemptinne et al (2000) a, Hemptinne et al (2000) b, Agarwala and Dixon (1992) - Heterospecific eggs and conspecific eggs painted with water extract from Coccinella septempunctata eggs , Sato and Dixon (2004)**
			x					**x**	**Hemptinne et al (2000) b**
		Harmonia axyridis	**x**						**Katsanis et al (2013), Sato and Dixon (2004), Ware et al (2009), Burgio et al (2002)**
			x					**x**	**Sato and Dixon (2004), Ware et al (2009)**

Adalia decempunctata	Adult	*Adalia bipunctata*	**x**						**Agarwala and Dixon (1992)**
Adalia decempunctata	Adult	*Ceratomegilla undecimnotata*	**x**						**Agarwala and Dixon (1992)**
		Coccinella septempunctata	**x**						**Agarwala and Dixon (1992)**
	Larvae	*Adalia bipunctata*	**x**						**Agarwala and Dixon (1992)**
		Ceratomegilla undecimnotata		**x**					**Agarwala and Dixon (1992)**
		Coccinella septempunctata		**x**					**Agarwala and Dixon (1992)**
		Harmonia axyridis	**x**						**Katsanis et al (2013)**
Anatis ocellata	Larvae	*Harmonia axyridis*	**x**						**Katsanis et al (2013)**
Aphidecta obliterata	Larvae	*Harmonia axyridis*	**x**						**Katsanis et al (2013)**
Calvia decemguttata	Larvae	*Harmonia axyridis*		**x**					**Katsanis et al (2013)**

Calvia quatuordecimguttata	Larvae	*Harmonia axyridis*	**x**						**Katsanis et al (2013)**
Ceratomegilla undecimnotata	Adult	*Adalia bipunctata*	**x**						**Agarwala and Dixon (1992)**
		Adalia decempunctata	**x**						**Agarwala and Dixon (1992)**
		Coccinella septempunctata		**x**					**Agarwala and Dixon (1992)**
	Larvae	*Adalia bipunctata*	**x**						**Agarwala and Dixon (1992)**
		Adalia decempunctata		**x**					**Agarwala and Dixon (1992)**
		Coccinella septempunctata		**x**					**Agarwala and Dixon (1992)**
Coccinella septempunctata	Adult	*Adalia bipunctata*	**x**						**Agarwala and Dixon (1992)**

		Adalia decempunctata	**x**						**Agarwala and Dixon (1992)**
		Ceratomegilla undecimnotata		**x**					**Agarwala and Dixon (1992)**
Coccinella septempunctata	Larvae	*Adalia bipunctata*		**x**					**Hemptinne et al (2000) a, Hemptinne et al (2000) b, Sato and Dixon (2004)**
			x						**Agarwala and Dixon (1992) - Heterospecific eggs and conspecific eggs painted with water extract from Coccinella septempunctata eggs**
		Adalia decempunctata	**x**						**Agarwala and Dixon (1992)**
		Ceratomegilla undecimnotata		**x**					**Agarwala and Dixon (1992)**

		Harmonia axyridis		**x**					**Hironori & Katsuhiro (1997), Hemptinne et al (2010)**
			x						**Katsanis et al (2013)**
Coccinella septempunctata	Larvae	*Harmonia axyridis*	**x**					**x**	**Kajita et al (2010), Rieder et al (2008), Sato and Dixon (2004)**
		Propylea japonica						**x**	**Sato et al (2008)**
Coccinella transversalis	Larvae	*Propylea dissecta*	**x**						**Omkar and Gupta (2004)**
Coccinella unidecimpunctata	Larvae	*Harmonia axyridis*		**x**					**Noia et al (2007)**
Coleomegilla maculata	Adult	*Cycloneda munda*		**x**					**Cottrel (2007)**
		Harmonia axyridis		**x**					**Cottrel (2007), Smith and Gardiner (2013) a&b**

			x						**Cottrell (2005)**
		Hippodamia convergens		**x**					**Cottrel (2007)**
		Olla v-nigrum		**x**					**Cottrell (2005), Cottrel (2007)**
Coleomegilla maculata	Larvae	*Cycloneda munda*		**x**					**Cottrel (2007)**
		Harmonia axyridis		**x**					**Cottrel (2007), Smith and Gardiner (2013) a & b**
			x						**Cottrell (2004)**
		Hippodamia convergens		**x**					**Cottrel (2007), Smith and Gardiner (2013) a**
		Olla v-nigrum		**x**					**Cottrel (2007)**
Cycloneda munda	Adult	*Coleomegilla maculata*		**x**					**Cottrel (2007)**
		Harmonia axyridis	**x**						**Cottrel (2007)**

		Hippodamia convergens	**x**						**Cottrel (2007)**
		Olla v-nigrum	**x**						**Cottrel (2007)**
	Larvae	*Coleomegilla maculata*		**x**					**Cottrel (2007)**
		Harmonia axyridis	**x**						**Cottrel (2007)**
		Hippodamia convergens	**x**						**Cottrel (2007)**
Cycloneda munda	Larvae	*Olla v-nigrum*	**x**						**Cottrel (2007)**
Harmonia axyridis	Adult	*Adalia bipunctata*		**x**					**Burgio et al (2002)**
		Coleomegilla maculata		**x**					**Cottrell (2005), Cottrel (2007)**
		Cycloneda munda		**x**					**Cottrel (2007)**
		Hippodamia convergens		**x**					**Cottrel (2007)**
		Olla v-nigrum		**x**					**Cottrell (2005), Cottrel**

									(2007)
	Larvae	*Adalia bipunctata*		**x**					**Burgio et al (2002)**
				x		**x**			**Ware et al (2009)**
				x			**x**		**Katsanis et al (2013)**
			x					**x**	**Sato and Dixon (2004)**
		Adalia decempunctata		**x**			**x**		**Katsanis et al (2013)**
		Anatis ocellata	**x**				**x**		**Katsanis et al (2013)**
		Aphidecta obliterata		**x**			**x**		**Katsanis et al (2013)**
Harmonia axyridis	Larvae	*Calvia decemguttata*		**x**			**x**		**Katsanis et al (2013)**
		Calvia quatuordecimguttata	**x**				**x**		**Katsanis et al (2013)**
		Coccinella septempunctata		**x**		**x**			**Sloggett et al (2009)**
				x			**x**		**Kajita et al (2010), Katsanis et al (2013)**

			x				**x**		**Rieder et al (2008), Sato and Dixon (2004)**
		Coccinella unidecimpunctata		**x**					**Noia et al (2007)**
		Coleomegilla maculata		**x**					**Cottrel (2007)**
				x			**x**		**Cottrell (2004)**
				x				**x**	**Sloggett et al (2009)**
		Cycloneda munda		**x**					**Cottrel (2007)**
				x			**x**		**Sloggett et al (2009)**
Harmonia axyridis	Larvae	*Hippodamia convergens*		**x**					**Cottrel (2007)**
				x		**x**			**Sloggett et al (2009)**
		Hippodamia undecimnotata		**x**			**x**		**Katsanis et al (2013)**

		Hippodamia variegata		**x**			**x**		**Katsanis et al (2013)**
		Oenopia conglobata		**x**			**x**		**Katsanis et al (2013)**
		Olla v-nigrum		**x**					**Cottrel (2007)**
				x		**x**			**Cottrell (2004)**
		Propylea japonica				**x**			**Sato et al (2008)**
		Propylea quatuordecempunct-ata		**x**			**x**		**Katsanis et al (2013)**
Hippodamia convergens	Adult	*Coleomegilla maculata*		**x**					**Cottrel (2007)**
		Cycloneda munda		**x**					**Cottrel (2007)**
		Harmonia axyridis		**x**					**Cottrel (2007)**
Hippodamia convergens	Adult	*Olla v-nigrum*		**x**					**Cottrel (2007)**
	Larvae	*Coleomegilla*		**x**					**Cottrel (2007)**

		maculata							
		Cycloneda munda		**x**					**Cottrel (2007)**
		Harmonia axyridis		**x**					**Cottrel (2007)**
		Olla v-nigrum	**x**						**Cottrel (2007)**
Hippodamia undecimnotata	Larvae	*Harmonia axyridis*	**x**						**Katsanis et al (2013)**
Hippodamia variegata	Larvae	*Harmonia axyridis*		**x**					**Katsanis et al (2013)**
Oenopia conglobata	Larvae	*Harmonia axyridis*		**x**					**Katsanis et al (2013)**
Olla v-nigrum	Adult	*Coleomegilla maculata*		**x**					**Cottrell (2005), Cottrel (2007)**
		Cycloneda munda		**x**					**Cottrel (2007)**
		Harmonia axyridis		**x**					**Cottrel (2007)**
			x						**Cottrell (2005)**
Olla v-nigrum	Adult	*Hippodamia convergens*		**x**					**Cottrel (2007)**

	Larvae	*Coleomegilla maculata*		**x**					**Cottrel (2007)**
				x			**x**		**Cottrell (2004)**
		Cycloneda munda		**x**					**Cottrel (2007)**
		Harmonia axyridis	**x**						**Cottrel (2007)**
			x					**x**	**Cottrell (2004)**
		Hippodamia convergens		**x**					**Cottrel (2007)**
Propylea dissecta	Larvae	*Coccinella transversalis*	**x**						**Omkar and Gupta (2004)**
Propylea quatuordecempunct-ata	Larvae	*Harmonia axyridis*	**x**						**Katsanis et al (2013)**

Appendix II

Table summarising the extent to which oviposition is deterred in female ladybirds by the presence and/or the tracks of other ladybird adults or larvae, either conspecifics or heterospecifics. Species vary the extent to which they are deterred from laying and the extent to which they deter laying, but overall the strongest deterrence effect is from conspecifics.

Ovipositing species	***Deterrent species***					
	Conspecific/ Heterospecific	**Species**	**Life stage**	**Individuals /Tracks**	**Ovipostion deterred?**	**Reference**
Adalia bipunctata	Conspecific	*Adalia bipunctata*	Adult	Individuals	Y	Hemptinne et al (1992)
				Individuals	N	Kajita et al (2006)
				Tracks	Y	Doumbia et al (1998)
			Larvae	Individuals	Y	Hemptinne et al (1992)
				Track extract	Y	Hemptinne et al (2001)

				Tracks	Y	Hemptinne et al (2001), Doumbia et al (1998), Frechette et al (2004), Laubertie et al (2006), Martini et al (2013), Frechette et al (2003)
Adalia bipunctata	Heterospecific	*Adalia decempunctata*	Larvae	Individuals	N	Hemptinne et al (1992)
				Tracks	N	Doumbia et al (1998)
					Y	Magro et al (2007)
		Coccinella septempunctata	Adult	Individuals	Y	Kajita et al (2006)
			Larvae	Individuals	N	Hemptinne et al (1992)
				Tracks	Y	Magro et al (2007)
					N	Doumbia et al (1998)
		Harmonia axyridis	Adult	Individuals	Y	Kajita et al (2006)
Adalia decempunctata	Conspecific	*Adalia decempunctata*	Larvae	Tracks	Y	Magro et al (2007)
	Heterospecific	*Adalia bipunctata*	Larvae	Tracks	Y	Magro et al (2007)
		Coccinella septempunctata	Larvae	Tracks	Y	Magro et al (2007)

Anegleis cardoni	Conspecific	*Anegleis cardoni*	Adults	Individuals	Y	Mishra et al (2012)
			Pupae	Individuals	N	Mishra et al (2012)
			Larvae	Individuals	Y	Mishra et al (2012)
			Eggs	Individuals	Y	Mishra et al (2012)
Aphidecta obliterata	Conspecific	*Aphidecta obliterata*	Larvae	Tracks	Y	Oliver et al (2007)
	Heterospecific	*Adalia bipunctata*	Larvae	Tracks	N	Oliver et al (2007)
Ceratomegilla undecimnotata	Conspecific	*Ceratomegilla undecimnotata*	Larvae	Tracks	Y	Ruzicka (2003)
	Heterospecific	*Leis dimidiata*	Larvae	Tracks	Y	Ruzicka (2003)
Cheilomenes sexmacultata	Conspecific	*Cheilomenes sexmacultata*	Adults	Individuals	Y	Mishra et al (2012)
			Pupae	Individuals	N	Mishra et al (2012)
			Larva	Individuals	Y	Mishra et al (2012)
				Tracks	Y	Ruzicka (2006)
			Eggs	Individuals	Y	Mishra et al (2012)
	Heterospecific	*Ceratomegilla undecimnotata*	Larvae	Tracks	Y	Ruzicka (2006)

		Cycloneda limbifer	Larvae	Tracks	Y	Ruzicka (2006)
		Harmonia dimidiata	Larvae	Tracks	N	Ruzicka (2006)
Coccinella septempunctata	Conspecific	*Coccinella septempunctata*	Adult	Individuals	Y	Hemptinne et al (1993)
					N	Mishra et al (2012)
Coccinella septempunctata	Conspecific	*Coccinella septempunctata*	Pupae	Individuals	N	Mishra et al (2012)
			Larvae	Individuals	N	Mishra et al (2012), Hemptinne et al (1993)
				Tracks	Y	Magro et al (2007), Ruzicka (1997), Ruzicka (2001), Ruzicka et al (2002)
					N	Doumbia et al (1998)
			Eggs	Individuals	Y	Mishra et al (2012)
					N	Hemptinne et al (1993)
	Heterospecific	*Adalia bipunctata*	Larvae	Tracks	Y	Doumbia et al (1998)
					N	Magro et al (2007)

		Adalia decempunctata	Larvae	Tracks	N	Magro et al (2007)
		Cycloneda limbifer	Larvae	Tracks	N	Ruzicka (2001)
		Leis dimidiata	Larvae	Tracks	N	Ruzicka (2001)
		Semiadalia undecimnotata	Larvae	Tracks	N	Ruzicka (2001)
Coccinella transversalis	Conspecific	*Coccinella transversalis*	Adults	Individuals	Y	Mishra et al (2012)
			Pupae	Individuals	N	Mishra et al (2012)
			Larvae	Individuals	Y	Mishra et al (2012)
			Eggs	Individuals	Y	Mishra et al (2012)
Coleomegilla maculata	Conspecific	*Coleomegilla maculata*	Larvae	Tracks	Y	Michaud and Jyoti (2007)
Cycloneda limbifer	Conspecific	*Cycloneda limbifer*	Larvae	Tracks	Y	Ruzicka (2001), Ruzicka et al (2002), Ruzicka (2003)

	Heterospecific	*Coccinella septempunctata*	Larvae	Tracks	N	Ruzicka (2001)
		Leis dimidiata	Larvae	Tracks	Y	Ruzicka (2001)
		Semiadalia undecimnotata	Larvae	Tracks	Y	Ruzicka (2001)
Harmonia axyridis	Conspecific	*Harmonia axyridis*	Adult/Larvae	Faeces	Y	Agarwala et al (2003)
			Larvae	Tracks	Y	Yasuda et al (2000)
	Heterospecific	*Coccinella septempunctata*	Larvae	Tracks	N	Yasuda et al (2000)
		Propylea japonica	Adult/Larvae	Faeces	N	Agarwala et al (2003)
Hippodamia convergens	Conspecific	*Hippodamia convergens*	Larvae	Tracks	Y	Michaud and Jyoti (2007)
Leis dimidiata	Conspecific	*Leis dimidiata*	Larvae	Tracks	N	Ruzicka (2001)
	Heterospecific	*Coccinella septempunctata*	Larvae	Tracks	N	Ruzicka (2001)
		Cycloneda limbifer	Larvae	Tracks	N	Ruzicka (2001)
		Semiadalia undecimnotata	Larvae	Tracks	N	Ruzicka (2001)
Propylea dissecta	Conspecific	*Propylea dissecta*	Adult	Tracks	Y	Mishra and Omkar (2006)

			Adults	Individuals	Y	Mishra and Omkar (2006), Mishra et al (2012)
			Pupae	Individuals	Y	Mishra et al (2012)
			Larvae	Individuals	Y	Mishra et al (2012)
			Eggs	Individuals	Y	Mishra et al (2012)
Propylea japonica	Conspecific	*Propylea japonica*	Adult/Larvae	Faeces	Y	Agarwala et al (2003)
	Heterospecific	*Harmonia axyridis*	Adult/Larvae	Faeces	Y	Agarwala et al (2003)
Semiadalia undecimnotata	Conspecific	*Semiadalia undecimnotata*	Larvae	Tracks	Y	Ruzicka (2001), Ruzicka et al (2002)
	Heterospecific	*Coccinella septempunctata*	Larvae	Tracks	N	Ruzicka (2001)
		Cycloneda limbifer			N	Ruzicka (2001)
		Leis dimidiata			Y	Ruzicka (2001)

Appendix III

Hue was excluded from analysis in both chapters 4 and 5, due firstly to the low UV reflectance of eggs, which influenced the reliability of the measures, and secondly to the high correlation of hue measures with both luminance and saturation.

Calculation of hue

Prior to calculations of hue, single cone catch values (for ladybird vision, Lin *et al.* 1992) were converted into proportions to remove absolute variation in brightness (Endler & Mielke 2005). The proportional cone catch values were then converted into two colour space coordinates (X, Y), giving each individual a location of colour in two dimensional colour space (Kelber *et al.* 2003; Endler & Mielke 2005). Recent methods of calculating hue have used principal component analysis to decide the type of colour channels (ratios) that would be most appropriate to explain colour variation, with colour channels broadly analogous to opponent colour channels in visual processing (Komdeur *et al.* 2005; Spottiswoode & Stevens 2010; Stevens 2011). However, the poor UV reflectance of ladybird eggs resulted in the presence of zero and negative values in the dataset, indicating that the UV component was no informative 'noise'. It also meant that hue equations created based on PCA results were not bounded and therefore were neither biologically plausible nor statistically useful e.g. when calculating hue 1, (mw/sw)/uv, as many values of uv are zero it means that mathematically both lw and mw can increase to infinity. Consequently a proportion of each wavelength was used to represent a form of

‘hue’, though in actuality it will also contain properties corresponding to changes in both saturation and hue (Montgomerie & McGraw, 2006); Hue 1: uv/(uv+sw+mw), Hue 2: sw/(uv+sw+mw) and Hue 3: mw/(uv+sw+mw).

Exclusion of hue from analysis

As in previous studies (Winters *et al.* 2014) our a priori expectation was that there would be no difference in the type of pigment in eggs, and therefore the type of colour of eggs (i.e. hue), either between treatments or between female morphs, but that there would be differences in pigment quantity and therefore in luminance and saturation. Due to this and the strong correlation of the calculated values of hue with both luminance and saturation (Table 1) hue was excluded from the analysis.

	X_1^2	p
Luminance vs Saturation	**15.909**	**<0.001**
Luminance vs Hue 1	**23.43**	**<0.001**
Luminance vs Hue 2	**6.6136**	**0.01**
Luminance vs Hue 3	**12.16**	**<0.001**
Saturation vs Hue 1	**1035.4**	**<0.001**
Saturation vs Hue 2	**11.734**	**<0.001**
Saturation vs Hue 3	**2062.3**	**<0.001**

Table 1. Correlations (glmm) between egg luminance, saturation and three hue values.

Appendix IV

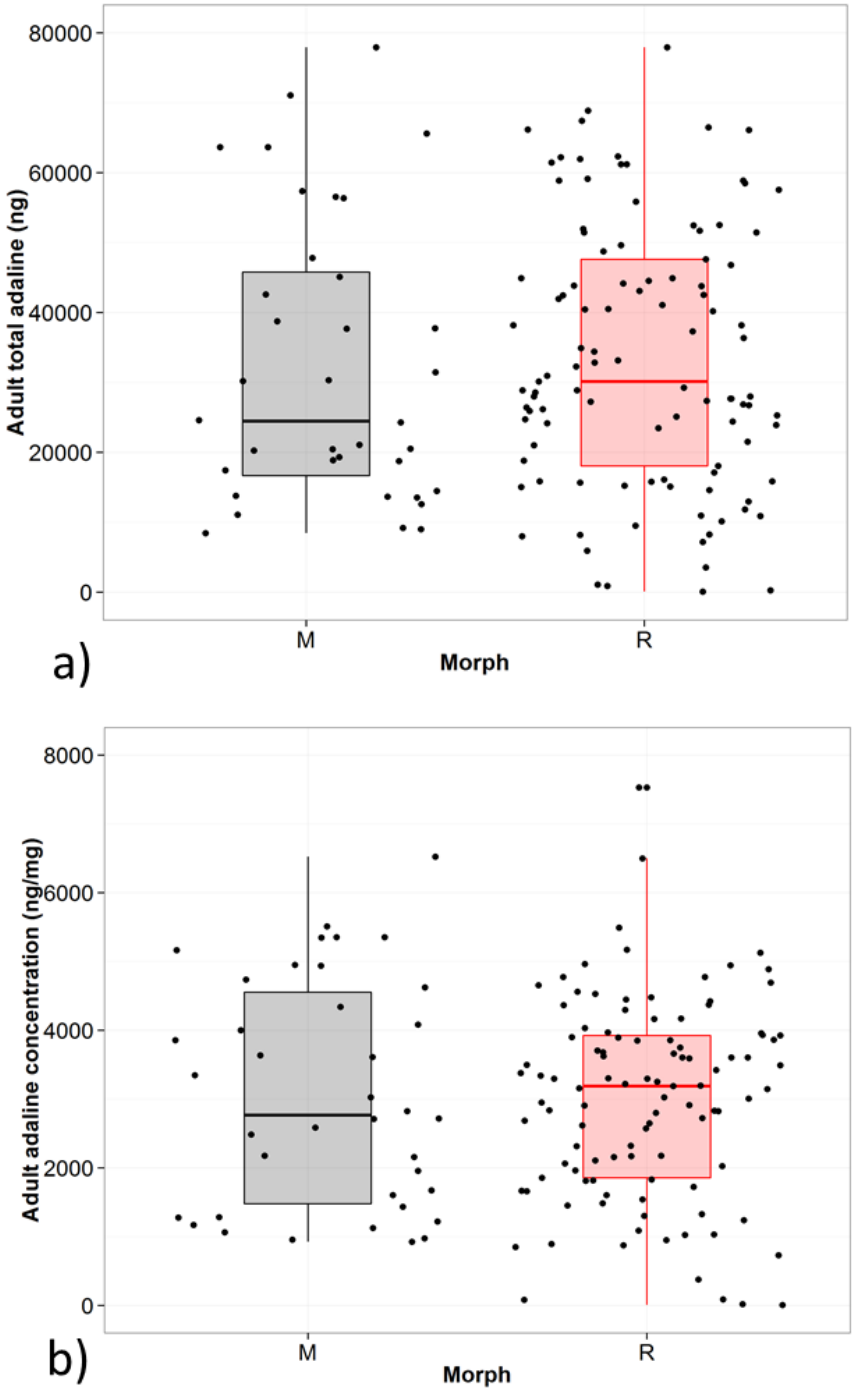

Figure 1. Lack of difference between a) total toxin content ($t_{1,139}$=-0.77, p=0.44 (NS)) and b) toxin concentration ($t_{1,139}$=-0.80, p=0.42 (NS)) of melanic (black; M) and typica (red; R) morph *A.bipunctata* adults. Data analysed using general linear model with adult adaline/adult adaline concentration as the response variable and morph (M or R), sex (M or F), and age as explanatory variables.

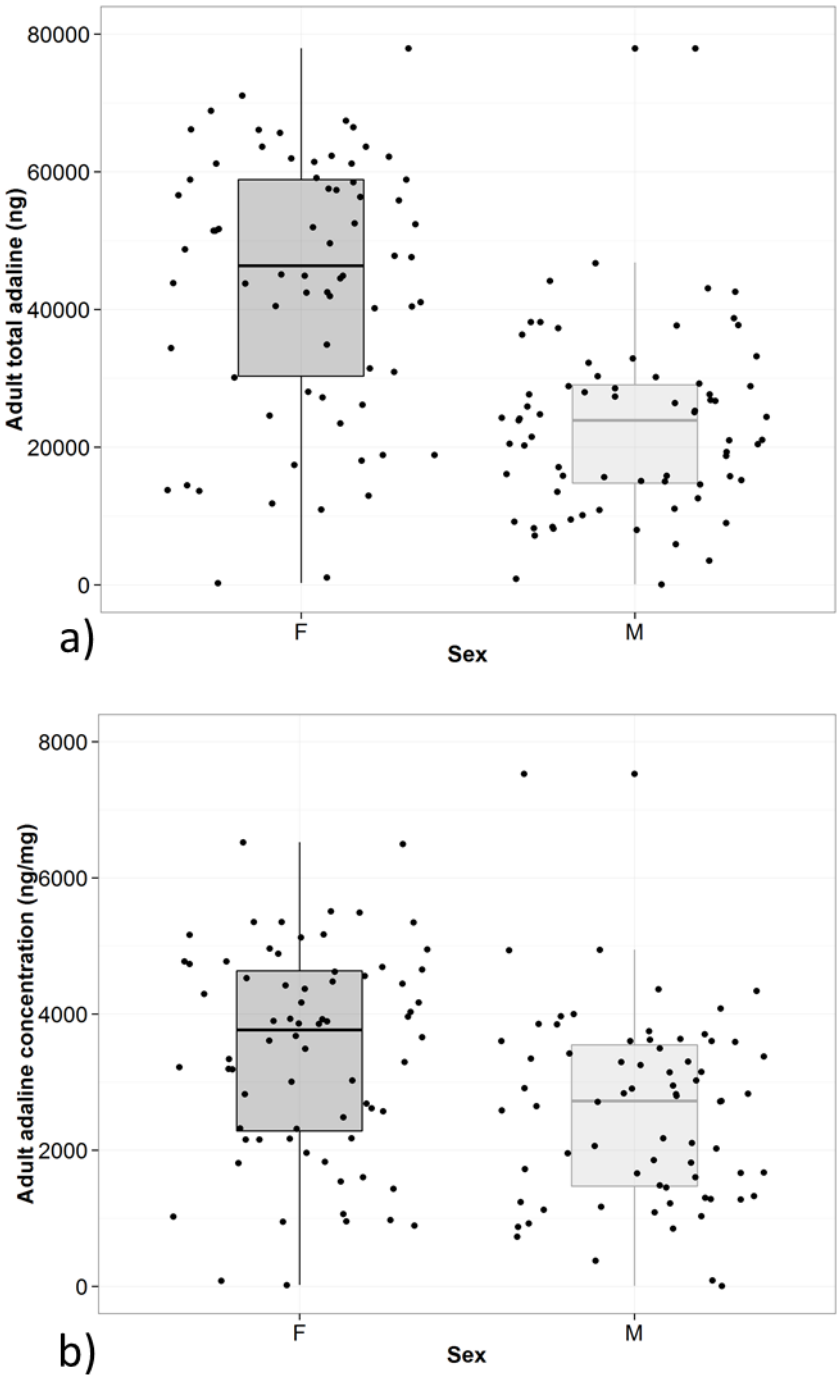

Figure 2. Higher a) total toxin content ($t_{1,140}$=-0.81, p<0.001) and b) toxin concentration ($t_{1,140}$=-3.90, p<0.001) of female (F) than male (M) *A.bipunctata* adults. Data analysed using general linear model with adult adaline/adult adaline concentration as the response variable and sex (M or F) and age as explanatory variables.

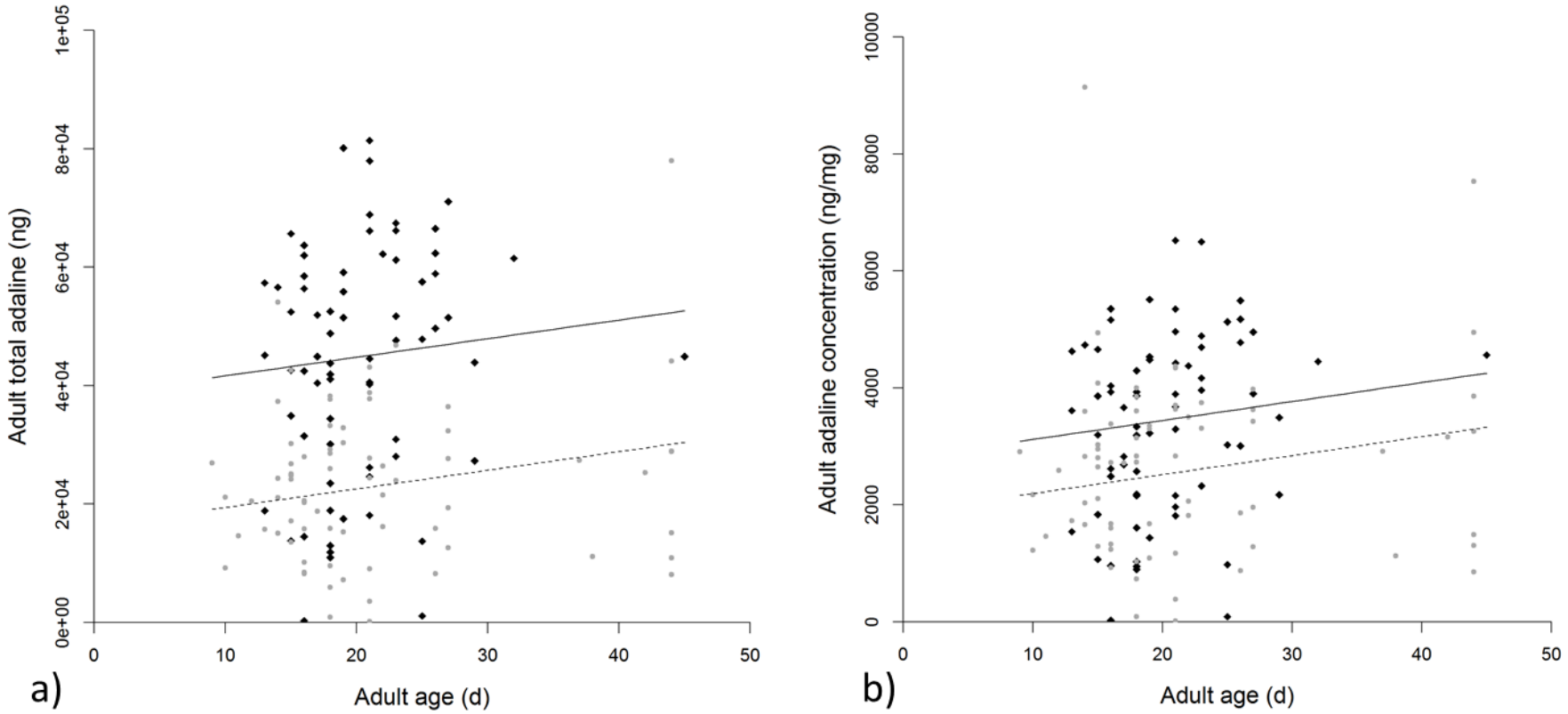

Figure 3. Effect of age on the a) total toxin content ($t_{1,140}$=1.73, p=0.085 (NS)) and b) toxin concentration ($t_{1,140}$=2.08, p=0.039) of female (black points and solid trend line) and male (grey points and dashed line) *A.bipunctata* adults. Data analysed using general linear model with adult adaline/adult adaline concentration as the response variable and sex (M or F) and age as explanatory variables.

Appendix V

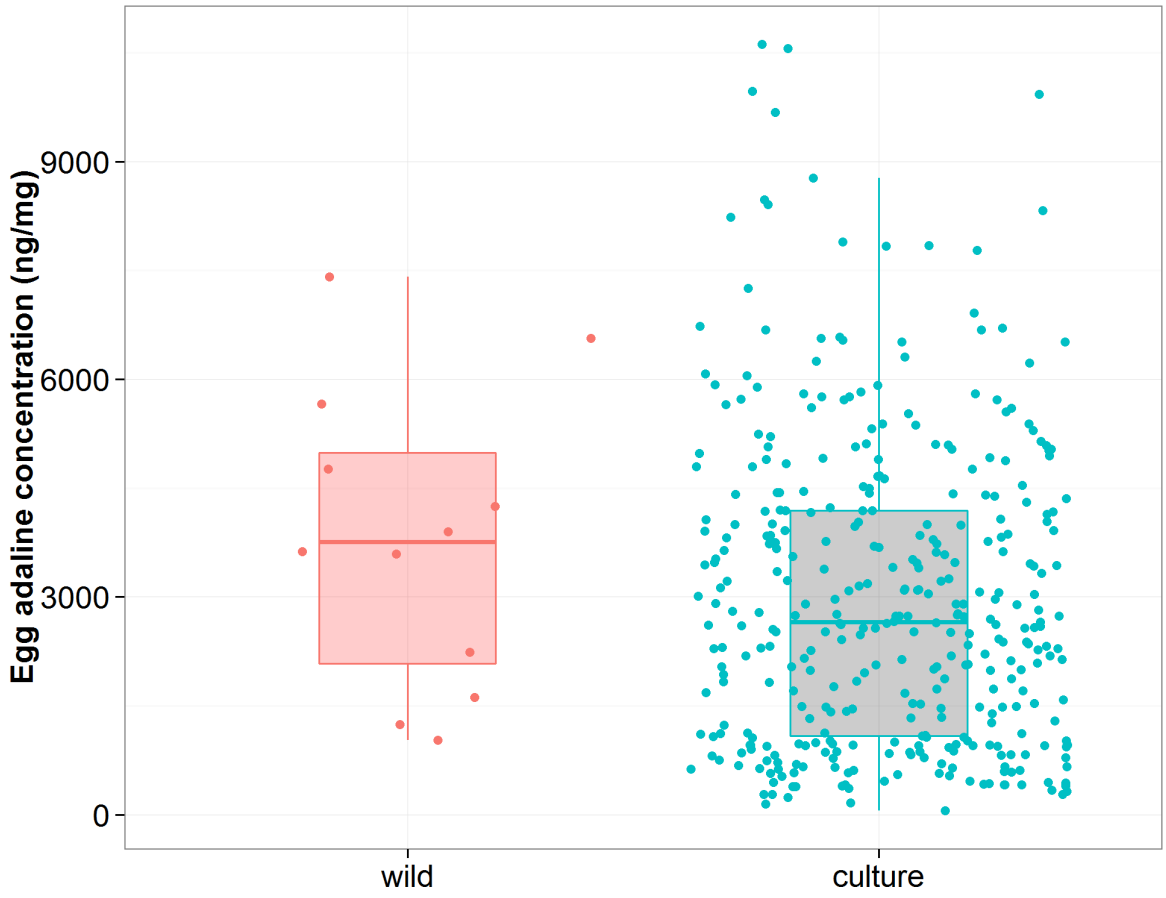

Figure 1. Lack of significant difference between adaline concentrations (ng/mg) laid by wild females and those taken from culture (X^2_1= 1.1, p= 0.3; linear mixed effects model with sqrt of egg adaline as the response variable, adult origin (wild or culture) as fixed and female ID as random explanatory variables)

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